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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/68, 15/63	A1	(11) International Publication Number: WO 91/09129 (43) International Publication Date: 27 June 1991 (27.06.91)

(21) International Application Number: PCT/DK90/00332

(22) International Filing Date: 18 December 1990 (18.12.90)

(30) Priority data:
6396/89 18 December 1989 (18.12.89) DK

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.

Published
With international search report.

(54) Title: STABLE INTEGRATION OF DNA IN BACTERIAL GENOMES

(57) Abstract

A bacterial cell which in its genome carries an integrated non-replicative DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of the cell, and (3) an origin of replication, said DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication.

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STABLE INTEGRATION OF DNA IN BACTERIAL GENOMES

FIELD OF INVENTION

The present invention relates to a bacterial cell which comprises a DNA construct integrated in its genome, a DNA construct intended for integration in the genome of a bacterial cell, a plasmid vector comprising the DNA construct, and methods of integrating the DNA construct into bacterial genomes.

BACKGROUND OF THE INVENTION

When, for the purpose of producing a desired polypeptide by recombinant DNA procedures, bacterial cells are transformed with a recombinant plasmid vector which carries inserted genetic information coding for said polypeptide, it has often been observed that such plasmids become unstable even though they may, in themselves, be stably inherited in the cell. This instability may either take the form of unstable maintenance of the plasmid in the cells so that the plasmid will eventually be lost from a cell population, or so that the DNA coding for the protein in question may be deleted from the plasmid. A traditional way of solving the former problem has been to grow the transformed cells under selection pressure, that is, typically in the presence of an antibiotic to which the cells in question have been made resistant due to the presence of a gene coding for a product mediating resistance to that antibiotic on the plasmid transformed to the cells. This approach, however, is neither economically feasible in large-scale production due to the high cost of the antibiotics in question, nor is it desirable for environmental reasons. The use of antibiotics in culture media also makes it more difficult to obtain product approval from health authorities and the like.

It has previously been suggested to stabilise plasmids by inserting into them a DNA s quence encoding a partitioning

function which ensures the even distribution of plasmids to progeny cells on cell division. An alternative method of achieving the stable inheritance of cloned DNA sequences is to provide for the integration of such DNA sequences in the genome of the host bacterium. Integration of DNA sequences present on plasmid vectors may take place by the so-called "crossing-over" procedure, e.g. as described by A. Campbell, Advances Genet. 11, 1962, pp. 101-145. According to this procedure, the plasmid vector is provided with a DNA sequence which is homologous to a region on the bacterial genome, or alternatively with two homologous sequences placed on either side of the heterologous DNA sequence to be integrated. In a subsequent recombination event, the homologous sequence and adjacent sequences on the vector are integrated into the host genome at the region of homology.

In some cases, however, is has been found that the integrated DNA sequences are deleted from the cells in the absence of selection pressure, for instance by a similar type of homologous recombination event as that responsible for the integration of the DNA. In particular, it has previously been observed that recombination between homologous DNA sequences is stimulated in the proximity of replicative DNA present on or near the DNA integrated in the host cell genome, cf. Ph. Noirot et al., J. Mol. Biol. 196, 1987, pp. 39-48; and M. Young and S.D. Ehrlich, J. Bacteriol. 171(5), May 1989, pp. 2653-2656.

An object of the present invention is therefore to provide stable integration of DNA sequences into genomic DNA, e.g. the chromosome, of bacterial host cells.

SUMMARY OF THE INVENTION

The present invention is based on the finding that stable integration of DNA sequences into the genome of host bacteria may be obtained by avoiding the presence f a functional plasmid replication system in the integrated DNA.

Accordingly, in one aspect, the present invention relates to a bacterial cell which in its genome carries an integrated non-replicative DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of the cell, and (3) an origin of replication, said DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication.

In another aspect, the present invention relates to a DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the DNA construct, and (3) an origin of replication, said DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication.

In the present context, the term "non-replicative DNA construct" is intended to mean a DNA sequence which is unable to replicate autonomously and which is therefore replicated together with the host cell genome. The genome comprises the chromosome and stably inherited extrachromosomal elements. The term "DNA sequence of interest" is used to indicate a sequence which may code for a desired RNA or protein product (heterologous or native to the host cell) or which may in itself provide the host cell with a desired property, e.g. a mutant phenotype as described below.

The homologous DNA sequence may typically be one derived from the genome of the host cell, and may be homologous with a region of the genome which is not essential for the survival or proper functioning of the host cell. On the other hand, the homologous DNA sequence may also be so selected that integration of the DNA construct of the invention by homologous recombination will lead to a cell expressing a mutant phenotype (which may then serve as a marker f r selection of cells in which the DNA construct has b en

integrated), for instance if the DNA construct is integrated within a transcription unit disrupting this so that one or more gene(s) contained within the transcription unit are consequently not expressed. The homologous DNA sequence may alternatively be one which is not native to the host genome but which has been cloned from another organism or which has been synthesized and subsequently introduced into the host genome by any convenient process, e.g. crossing-over, prior to the integration of the DNA construct of the invention. The homologous DNA sequence may be one which comprises or consists essentially of the DNA sequence of interest, whether native or foreign to the host cell in question (for instance native to the cell in cases where it is desired to amplify the copy number of the DNA sequence of interest in the cell, vide below). It should be noted that, in the present context, the term "homologous" may be defined as a sequence identity of at least 9 consecutive base pairs.

DETAILED DISCLOSURE OF THE INVENTION

Although, according to the invention, the stable integration of DNA into bacterial genomes has been demonstrated for plasmids with a particular type of replication system (the so-called rolling circle replication, vide below), it is currently expected that any plasmids which replicate by a mechanism where one or more trans-acting factors (i.e. RNA or protein factors) are required to initiate replication from cis-acting sequences on the plasmid (such cis-acting DNA sequences are collectively termed the origin of replication) will be useful for the present purpose. Factors which are necessary for plasmid replication will be termed replication factors in the following. When the DNA construct lacks a functional gene coding for a replication factor required by the origin of replication contained on said DNA construct, no active replication factor is produced and, consequently, no replication is initiated from the origin.

In order to obtain a DNA construct of the invention which lacks a functional gene encoding a required replication factor, one may either delete the entire gene or modify it in such a way that it encodes an inactive replication factor. Such modification of the gene may be carried out in a manner known per se by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by similar modifications of transcriptional or translational start or stop signals.

The replication system outlined above may be utilised in a method of constructing a bacterial cell of the invention. In one embodiment of the method, a plasmid vector for the present purpose termed a parental vector, is initially constructed. The parental vector comprises (i) a first origin of replication; (ii) one or more functional genes encoding the replication factor(s) required for replication from said first origin of replication; (iii) a second origin of replication in the same orientation as the first origin of replication; (iv) a DNA sequence of interest, and (v) a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the vector, said parental vector lacking a functional gene encoding a replication factor required for replication from the second origin of replication in the region between the second and the first origin of replication (in the above-mentioned order). It should be noted that, in the present context, the term "plasmid" is also intended to denote a bacteriophage or other DNA molecule capable of functioning as an autonomously replicating extrachromosomal element.

According to the invention, this parental vector may then be transformed into bacterial cells, and the transformed cells are cultured under conditions permitting replication of the vector.

Replication of the parental vector in th transformed cell gives rise to the formation of two different progeny vectors.

The first progeny vector comprises (i) a first origin of replication; (ii) one or more genes encoding the replication factor(s) required for replication from said origin. The second progeny vector comprises (iii) a second origin of replication; (iv) a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the plasmid vector, said second progeny vector lacking a functional gene coding for a replication factor required for replication from the second origin of replication carried on said second progeny vector. The formation of two progeny vector molecules from the parent vector may take place by different mechanisms; either as a result of the mode of replication of the plasmid, for instance rolling circle replication of single-stranded DNA plasmids (vide below), or as a result of homologous recombination between the DNA regions including and/or adjacent to the two origins of replication present on the plasmid vector.

When the second origin is located in the same orientation on the parent plasmid as the first origin, the various DNA sequences intended to be integrated into the bacterial genome and located downstream of the second origin, but upstream of the first origin (i.e. the DNA sequence of interest, and the DNA sequence which is homologous with a region of the genome of the cell) will be present on the second progeny vector following plasmid replication. Continued culturing of the transformed cells may spontaneously result in the integration of said second progeny vector into the bacterial genome by homologous recombination and loss of the first progeny vector from the cells with a certain frequency.

In order to facilitate selection for cells in which the second progeny vector has been integrated in the genome, this vector is preferably provided with a selectable marker. In this case, the cells may be cultured under selective conditions which is to say that only cells in which th selectable marker is maintained will survive. The selectable

marker may be a gene coding for a product which confers antibiotic resistance to the cell or which confers prototrophy to an auxotrophic strain (e.g. _dal_ genes introduced in a _dal_⁻ strain; cf. B. Diderichsen in _Bacillus: Molecular Genetics and Biotechnology Applications_, A.T. Ganesan and J.A. Hoch, Eds., Academic Press, 1986, pp. 35-46). Cells surviving under these conditions will either be cells containing the parental plasmid vector or containing both progeny vectors formed upon replication of the parental vector in the cell, or cells in which the second progeny vector comprising the DNA construct of the invention has been integrated. It has surprisingly been found that the parental vector and the first progeny vector are eventually lost whereas the second progeny vector comprising the DNA construct of the invention is spontaneously integrated in the host genome at a high frequency.

If it is desired to improve the efficiency with which integration of the DNA construct takes place, one may utilise, as the parental vector, a plasmid which is able to replicate under certain (permissive) conditions and unable to replicate under other (non-permissive) conditions. The plasmid may, for instance, be one which is temperature-sensitive for replication. Thus, in an embodiment of the method of the invention, the parental vector is one which is unable to replicate at increased temperatures, which yet permit growth of the host cell. The bacterial cells are initially cultured at a temperature permitting plasmid replication and formation of the two progeny vectors and subsequently, after integration of the second progeny vector, comprising the DNA construct of the invention, into the bacterial genome may have taken place, cultured at a temperature which does not permit plasmid replication so that the first progeny vector as well as the parental vector are lost from the cells. The cultivation at the non-permissive temperature is conducted under selective conditions to ensure that only cells containing the integrated DNA construct, including an appropriate selectable marker, will survive.

Another way of increasing the efficiency of integration and subsequent loss of the first progeny vector from the cells may be to treat the cells transformed with the parental vector with a plasmid-curing agent, e.g. novobiocin (Gadó, I. et al., 1987. Zbl. Bakt. Hyg. A. 265, 136-145), after culturing the host cells under selective conditions as described above.

It may be possible to employ replication origins from two different plasmids on the same parental vector, provided that these are sufficiently similar to each other to be functional with the same replication factor(s) which should be able to initiate replication from both the first and the second origin of replication. Alternatively, the plasmid vector should contain homologous regions in order to be able to undergo homologous recombination as described above. It is, however, preferred that the first origin of replication (and the gene coding for the replication factor associated therewith) is derived from the same plasmid as the second origin of replication in order to ensure that the replication mechanism on which the present invention is based will function optimally.

Out of practical considerations, it may be preferred to make the initial construction of the plasmid vector in an organism in which replication from the first and second origins of replication cannot be initiated or in which the rate of replication from these origins is very low. The plasmid vector may therefore be a shuttle vector provided with an additional origin of replication which makes the vector able to replicate in two different organisms. The additional origin of replication may, for instance, be one which is functional in _Escherichia coli_, this organism being well described and conventionally used for recombinant DNA experimentation and therefore suitable for constructing plasmids by recombinant DNA techniques. The shuttle vector may also compris an additional selectable marker, e.g. an antibiotic resistance gene, f r selection of the vector in _E._

coli. The additional origin of replicati n and selectable marker should preferably follow the first origin and/or the replication factor(s), but precede the second origin so that, on replication of the vector from the first and second origins, these additional sequences will be carried by the first progeny vector which is eventually lost from the bacterial cell transformed with the parental vector.

In an alternative method of producing the bacterial cell of the invention, host cells are transformed with a first DNA vector comprising a first origin of replication associated with a functional gene encoding a factor required for plasmid replication from said first origin of replication, and subsequently or simultaneously, by cotransformation, with a second DNA vector comprising a second origin of replication lacking an associated functional gene encoding a factor required for plasmid replication from the second origin of replication, as well as a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of the cell. The second DNA vector is also preferably provided with a selectable marker. The resulting cells containing the first and second DNA vectors are then cultured, preferably under selective conditions as described above, which eventually leads to integration of said second DNA vector into the bacterial genome by homologous recombination and loss of the first DNA vector for which there is no selection. As in the method described above initially employing a single plasmid vector, the first DNA vector may be one the replication of which is dependent on permissive and non-permissive conditions for culturing cells transformed with the vector. Thus in the method of the invention, the first DNA vector may be one which is unable to replicate at increased temperatures, which yet permit growth of the host cells, and the bacterial cells are initially cultured at a temperature permitting plasmid replication and subsequently, after integration of the second DNA vector into the bacterial genome, cultured at a temperature which does not permit plasmid replication so that the first DNA vector is lost from

the cells and under selective conditions so that only cells in which the second DNA vector is integrated, are able to survive. Similarly, the transformed cells may be treated with a plasmid-curing agent as described above.

An intermediate formed in both of the methods of constructing a cell containing the integrated non-replicative DNA construct discussed above is a bacterial cell which comprises a first DNA vector comprising a first origin of replication associated with one or more functional gene(s) encoding the factor(s) required for plasmid replication from said first origin of replication, and a second DNA vector comprising a second origin of replication , as well as a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of the cell, said second vector lacking a functional gene coding for a replication factor required for replicatin from the origin of replication carried on said second vector.

In order to obtain an origin of replication on the second DNA vector which lacks a functional gene coding for a replication factor required for replication from said origin, it is possible to delete this gene from the vector or to modify it in the ways indicated above. In particular when using two different origins of replication, the first DNA vector may also be provided with a gene coding for a replication factor required to initiate replication from the second origin of replication. In this way, replication of the second DNA vector depends on the second replication factor produced from the first DNA vector, and the second vector becomes non-replicative when the first vector is lost from the cell. However, the first and second origin of replication may also be derived from the same plasmid, in which case only one gene coding for an intact replication factor is required on the first vector.

Although, for the purpose of the present invention, the bacterial cell into which the plasmid vector or the first and

second DNA vector are transformed may be b th gram-negativ and gram-positive, it is preferably a cell of a gram-positive bacterium as it is generally easier to obtain extracellular expression of polypeptides from gram-positive organisms than from gram-negative ones. Thus, the bacterium may be of a strain belonging to the genus Bacillus or Streptomyces, in particular a strain of Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus subtilis or Streptomyces lividans.

The present invention is currently believed to be the only efficient method of providing stable homologous integration of DNA sequences of interest in genomes of bacteria which cannot be transformed by being made competent (or, at least, in which natural competence mechanisms have yet to be demonstrated), but which may be transformed by techniques including, for instance, protoplast formation or electroporation, e.g. certain strains of Bacillus licheniformis or Bacillus lentus. The present method is therefore of particular interest with respect to such organisms in which the transformation frequency is low, typically 10-50 transformants per μg of DNA (contrary to, e.g., transformation of competent E. coli or B. subtilis cells, where the number of transformants is typically on the order of $10^6 - 10^8$ per μg of DNA), which makes the sucessful transformation and stable integration of DNA in these organisms particularly important.

In the bacterial cell of the invention, the DNA sequence of interest is advantageously one which codes for a polypeptide of interest, and the present invention consequently further relates to a process for producing a polypeptide of interest, comprising culturing a bacterial cell according to the invention containing an integrated DNA sequence which codes for said polypeptide under conditions conducive to the production of the polypeptide and recovering the resulting polypeptide from the culture. The polypeptid pr duced by the

present process may b any polypeptide advantageously produced in bacteria such as an enzyme, e.g. a protease, amylase or lipase.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

A large family of plasmids from gram-positive bacteria replicate by the so-called "rolling circle replication" mechanism generating single-stranded DNA as a replication intermediate. Replication is initiated when a plasmid-encoded protein, Rep, recognizes an origin of replication sequence (the plus origin) and produces a nick in one of the DNA strands (the plus strand). The plus strand is then displaced, and a new plus strand is polymerized from the nick by 3'-OH extension. When the Rep protein subsequently recognizes a termination sequence (which overlaps the plus origin), it produces a second nick at the same position as the first one to generate a fully replicated strand and a single-stranded DNA monomer of the displaced strand the ends of which are ligated to form a circular molecule. Host factors then ensure the conversion of the single-stranded DNA molecule to double-stranded DNA (for a more detailed description of this type of plasmid, see A. Gruss and S.D. Ehrlich, Microbiological Reviews 53(2), June 1989, pp. 231-241). For the present purpose, plasmids with this replication system are termed single-stranded DNA plasmids.

It has surprisingly been found that the rolling circle replication mechanism may be utilised according to the invention to produce a bacterial cell according to the invention which harbours a DNA construct comprising, apart from a DNA sequence of interest and a DNA sequence which is homologous to a region of the genome of the cell,
a plus origin of replication from a single-stranded DNA plasmid, lacking a functional rep gene cognate to the plus origin of replication.

This bacterial cell may be construct d by the method of the invention using a parental plasmid vector which comprises (i) a first plus origin from a single-stranded DNA plasmid; (ii) a functional <u>rep</u> gene cognate to the first plus origin; (iii) a second plus origin from a single-stranded DNA plasmid in the same orientation as the first plus origin (iv) a DNA sequence of interest, and (v) a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the plasmid vector, said parental vector lacking, in the region between the second and the first plus origin in the same orientation as above, a functional <u>rep</u> gene cognate to the second plus origin.

On replication of the parental vector, the first and second progeny DNA vectors are formed, presumably by the following mechanism:

The Rep protein initiates replication by producing a nick at the first plus origin, and proceeds to make a nick at the second plus origin. The displaced strand is religated to form a first progeny vector comprising a first plus origin of replication from a single-stranded DNA plasmid and a functional <u>rep</u> gene cognate to the first plus origin. Similarly, the Rep protein proceeds from the second plus origin to make a nick at the first plus origin thus forming, on religation of the displaced strand and conversion of the single-stranded DNA to double-stranded DNA, a second progeny vector comprising a second plus origin of replication from a single-stranded DNA plasmid lacking a functional <u>rep</u> gene cognate to the second plus origin of replication, as well as a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of said cell. As the second progeny vector does not comprise a functional <u>rep</u> gene, replication of this molecule depends entirely on the Rep protein supplied <u>in trans</u> from either the first progeny vector or from the parental vector.

Alternatively, the two progeny vect rs c uld also be formed as a result of recombination between the homologous DNA regions including and/or adjacent to the two origins present on the parental vector.

A second progeny vector without a functional replication origin may be formed if the first plus origin in the parental plasmid described above is replaced by a small DNA fragment derived from the origin region which is sufficient to ensure termination of plasmid replication but too small to constitute a functional origin. Such fragments have been identified in pUB110 (Boe et al., 1989, J. Bacteriol., 171, 3366-3372) and in pC194 (Gros et al., 1987, EMBO J., 6, 3863-3869).

The bacterial cell may alternatively be constructed by a method of the invention comprising transforming the host cell with a first DNA vector comprising a first plus origin of replication from a single-stranded DNA plasmid associated with a functional rep gene, and subsequently or simultaneously, by cotransformation, transforming the host cell with a second DNA vector comprising a second plus origin of replication from a single-stranded DNA plasmid lacking a functional rep gene cognate to the second plus origin of replication, but comprising a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of said cell. The second DNA vector is maintained in the cell due to the presence of Rep protein supplied from the first DNA vector.

When the parental vector or second DNA vector comprises a modified rep gene, the second plus origin may precede or be located in the modified rep gene. As described above, the second plus origin may be derived from the same or a different plasmid as the first plus origin. In cases where the first and second plus origins are derived from different plasmids so that replication will not be initiated from both origins by means of the same Rep pr tein, the first DNA

vector may additionally contain a _rep_ gene encoding an active Rep protein capable of initiating replication from the second plus origin. The parental vector or second DNA vector may also comprise a selectable marker as described above.

In a favoured embodiment for promoting the integration process, a vector may be employed the replication of which is dependent on permissive conditions, including the temperature at which host cells are cultured. Thus, when a host bacterium containing the first and second DNA vectors is cultured at the permissive temperature for plasmid replication, the Rep protein produced from the first DNA vector will serve to maintain the second DNA vector in the cell. However, at non-permissive temperatures at which the first DNA vector is unable to replicate, the first vector and consequently the Rep protein produced from it will be lost from the cell so that the second DNA vector is no longer able to replicate either. By continued cultivation under selection pressure, e.g. in the presence of an antibiotic, only those cells survive which in their genome contain the inserted DNA construct of the invention, including a gene coding for a selectable marker.

It should be noted that once the DNA construct has been integrated in the genome of the host cell, this may be cultured in the absence of selection pressure without consequent loss of the DNA construct or parts thereof from the cell. This is believed to be ascribable to the fact that the integrated DNA is incapable of autonomous replication, but is replicated together with the host genome. The lack of autonomous replication of the integrated DNA implies that there is no formation of the single-stranded DNA intermediate which is believed to be responsible for the recombination process whereby integrated DNA is excised from the host genome (cf. Ph. Noiret et al., _J. Mol. Biol._ _196_, 1987, pp. 39-48; and M. Young and S. D. Ehrlich, _J. Bacteriol._ _171_(5), May 1989, pp. 2653-2656).

It has been found possible to amplify the integrated DNA construct by culturing transformed cells under increased selection pressure, e.g. at increased concentrations of an antibiotic. It has previously been found (cf.) that in the absence of selection pressure such amplified copies are frequently lost from the cells. Contrary to this, the present invention provides a bacterial cell in which amplified copies of integrated DNA sequences may be stably maintained in host cells because, as explained above, the integrated DNA is non-replicative. Although the present invention has mainly been described above as suitable for the integration of heterologous DNA sequences, it should be noted that the present method is also suitable for obtaining an amplified copy number of a gene which is homologous to the host cell in order to increase its production of a specific gene product.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further illustrated with reference to the accompanying drawings wherein

Fig. 1 shows a restriction map of plasmid pDN3000,

Fig. 2 shows a restriction map of plasmid pE194,

Fig. 3 shows a restriction map of plasmid pPL1975,

Fig. 4 shows a restriction map of plasmid pSX120,

Fig. 5 shows a restriction map of plasmid pPL2002,

Fig. 6 shows a restriction map of plasmid pDN3060,

Fig. 7 shows a restriction map of plasmid pSJ1085,

Fig. 8 shows a restriction map of plasmid pUC19,

Fig. 9 shows a restriction map of plasmid pSJ1103,

Fig. 10 shows a restriction map of plasmid pSJ1130,

Fig. 11 shows a restriction map of plasmid pSJ1136,

Fig. 12 shows a restriction map of plasmid pSJ1137,

Fig. 13 shows a restriction map of plasmid pPL1484,

Fig. 14 shows a restriction map of plasmid pSJ1155,

Fig. 15 shows a restriction map of plasmid pSJ1157,

Fig. 16 shows a restriction map of plasmid pSJ1259,

Fig. 17 shows a restriction map of plasmid pDN2904,

Fig. 18 shows a restriction map of plasmid pSJ1139,

Fig. 19 shows a restriction map of plasmid pSJ1139a,

Fig. 20 shows a restriction map of plasmid pSJ1139b,

Fig. 21 shows a restriction map of plasmid pdn3020,

Fig. 22 shows a restriction map of plasmid pPL1878,

Fig. 23 shows a restriction map of plasmid pPL1896,

Fig. 24 shows a restriction map of plasmid pSJ993,

Fig. 25 shows a restriction map of plasmid pSJ1163,

Fig. 26 shows a restriction map of plasmid pSJ1136a,

Fig. 27 shows a restriction map of plasmid pSJ1163b;

Fig. 28 shows a r striction map of plasmid pSJ1259a;

Fig. 29 shows a restriction map of plasmid pSJ1555;

Fig. 30 shows a restriction map of plasmid pSJ1555a; and

Fig. 31 shows a restriction map of plasmid pSJ1555b.

In all figures, arrows denote the direction of transcription.

To improve readability the replicational origins (+ ori pUB110, + ori pE194, ori pUC19) are indicated by the actual start site for replication, even though a functional origin consists of a larger DNA region.

The invention is further described in the following examples which are not intended to be in any way limiting to the scope and spirit of the invention as claimed.

MATERIALS AND METHODS

Plasmids

pBD64: described in Gryczan et al., 1980.

pDN3060: A cloning vector derived from the Bacillus plasmid pDN1050 (Diderichsen, B., 1986) by insertion of synthetic oligonucleotides containing a number of useful restriction sites. The restriction map is shown in Fig. 6.

pDN2904: A derivative of the Bacillus plasmid pUB110 (Gryczan et al., 1978), containing both a chloramphenicol resistance gene and a kanamycin resistance gene. The restriction map is shown in Fig. 17.

pPL1484: A pUC19 (Yanisch-Perron et al., 1985) derivative containing a modified polylinker region into which was inserted a 1.4 kb BamHI fragment from pDN2904 containing the kanamycin resistance gene. The restriction map is shown in Fig. 13.

pPL1878: pDN1380 (described in Diderichsen and Christiansen, 1988) containing a 2.4 kb HaeII-SphI fragment encoding a Cyclodextrin Glycosyl Transferase (CGTase) originating from Thermoanaerobacter sp. ATCC 53627. The gene was initially cloned into the _E. coli_ plasmid pBR322 on a 12.8 kb EcoRI fragment (Starnes et al., 1989). The restriction map is shown in Fig. 22.

Strains:

E.coli SJ 6: a restriction-deficient derivative of MC1000 (Diderichsen et al., 1990)

Bacillus subtilis DN1885: an _amy_E, _amy_R2, _spo_$^+$, Pro$^+$ derivative of _B.subtilis_ 168. (Diderichsen et al., 1990).

Bacillus subtilis DN1686: A _spo_$^-$ derivative of DN1280 containing a chromosomal deletion in the _dal_ gene (Diderichsen, 1986).

Bacillus licheniformis ATCC 9789

Bacillus lentus NCIB 10309

Media:

TY:	Trypticase	20	g/l
	Yeast xtract	5	g/l
	$FeCl_2.4H_2O$	6	mg/l

	$MnCl_2.4H_2O$	1 mg/l
	$MgSO_4.7H_2O$	15 mg/l
	pH	7.3
TY9:	As TY media but the pH was adjusted to 8,5 by adding $NaHCO_3$ (0,1 M)	
TY9 agar :	Trypticase	20 g/l
	Yeast extract	5 g/l
	$FeCl_2.4H_2O$	6 mg/l
	$MnSO_4.4H_2O$	1 mg/l
	$MgSO_4.7H_2O$	15 mg/l
	Bacto agar	5 g/l
	Adjusted to pH 8,5 with $NaHCO_3$ (0,1 M)	
BPX:	Potato starch	100 g/l
	Barley flour	50 g/l
	BAN 5000 SKB	0.1g/l
	Sodium caseinate	10 g/l
	Soy Bean Meal	20 g/l
	Na_2HPO_4, 12 H_2O	9 g/l
	Pluronic	0.1g/l
LB agar:	Bacto-tryptone	10 g/l
	Bacto yeast extract	5 g/l
	NaCl	10 g/l
	Bacto agar	15 g/l
	Adjusted to pH 7.5 with NaOH	

GENERAL METHODS

The experimental techniques used to construct the plasmids were standard techniques within the field of recombinant DNA technology, cf. T. Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1982.

Restriction endonucleases were purchased fr m New England Biolabs and Boehringer Mannheim and used as recommended by the manufacturers. T4 DNA ligase was purchased from New England Biolabs and used as recommended by the manufacturer.

Preparation of plasmid DNA from all strains was conducted by the method described by Kieser, 1984.

Transformation of _E. coli_:
Cells of _E. coli_ were made competent and transformed as described by Mandel and Higa, 1970, or were transformed by electroporation as described in the manual for the BIO-RAD Gene Pulser electroporation apparatus.

Transformation of _B. subtilis_:
Competent cells were prepared and transformed as described by Yasbin et al., 1975.

Transformation of _B. licheniformis_:
Plasmids were introduced into _B. licheniformis_ by polyethylene glycol-mediated protoplast transformation as described by Akamatzu, 1984.

Transformation of _B.lentus._
Plasmids were introduced into _B.lentus_ by protoplast transformation according to a slightly modified procedure by Akamatzu (1984). The modifications were a higher pH in the regeneration medium e.g. the HCP 1,5 medium were buffered to pH 8,5 by adding 0,1 M $NaHCO_3$ to the medium.

EXAMPLE 1

Stable integration of a non-replicative DNA molecule in the Bacillus lentus chromosome.

Cloning of the Subtilisin 309 gene

The g ne coding for the protease designated subtilisin 309 was cloned from an isolate of the B.lentus strain NCIB 10309 as described in WO 89/06279. Further subcloning resulted in the plasmid pSX120, which contains the replication origin of pUB110, the chloramphenicol resistance gene (cat) from pC194, two promoters P_{AmyH} and P_{AmyQ} and the gene encoding for the subtilisin 309 protease. (See Fig.4 and International Patent Application No. PCT/DK90/00164)

Construction of the integration plasmid pPL2002.

Plasmid pDN3000 was constructed by restricting pUC19 (Yanisch-Perron et. al.) with EcoRI and inserting the following oligonucleotide sequence (prepared by the phosphoamidite method described by Beaucage and Caruthers, Tetrehedron letters 22, 1981, pp. 1859-1869, on an automatic DNA synthesizer)

AATTGATCAAGCTTTAAATGCATGCTAGCAACGCGGCCGCCAACCTCGAGATCTCATG

CTAGTTCGAAATTTACGTACGATCGTTGCGCCGGCGGTTGGAGCTCTAGAGTACTTAA

into the linearized pUC19 followed by ligation. The ligation mixture was then used to transform competent E.coli SJ6 cells and transformants were selected on LB plates containing 100 ug/ml ampicillin. The orientation of the inserted linker in pDN3000 is as indicated by the orientation of the restriction sites in Fig.1.

Plasmid pPL1975 was constructed by restricting pDN3000 with BglII followed by ligation of this linearized plasmid to the MboI fragment containing the DNA from position 1 to 1585 resulting from restriction of pE194 (Fig.2, Horinouchi and Weisblum) with MboI. The ligation mixture was then used to transform competent E.coli SJ6 cells and transformants were selected on LB plates containing 100 ug/ml ampicillin. The orientation of the connection of these two fragments is as

indicated in Fig.3. pPL1975 thus contains a functional E. coli replication origin and a pE194 DNA fragment comprising an intact plus origin (+ ori pE194) and a truncated repF gene (repF') (Villafane et al., 1987).

Plasmid pPL2002 (Fig.5) was constructed by restricting pPL1975 (Fig.3) by EcoRI and BamHI and ligating the linearized plasmid to the 3.3kb EcoRI (partial) , BglII fragment from pSX120 (Fig. 4) containing the subtilisin 309 gene and the cat resistance gene. The ligation mixture was then used to transform competent E. coli SJ6 cells and transformants were selected on LB plates containing 100 ug/ml ampicillin.

Stable integration of the pPL2002 plasmid into the chromosome of B.lentus.

An isolate of the B.lentus strain NCIB 10309 was transformed by protoplast transformation with the temperature sensitive plasmid pE194 (See fig.1) selecting for erythromycin resistance (5ug/ml) at 30^0C (permissive temperature). The resulting strain was denoted PL2156.

PL2156 was then protoplast transformed with the plasmid pPL2002 selecting for chloramphenicol resistance (8ug/ml) and erythromycin resistance (5ug/ml) at 30^0C resulting in the strain PL2157 containing the two plasmids pE194 and pPL2002. In these cells the replication of the plasmid pPL2002 completely depends on the presence of the plasmid pE194 which encodes the for the pPL2002 replication, indispensable replication protein repF.

The strain PL2157 was grown overnight in TY9 medium and dilutions were plated on TY9 plates at 45^0C (nonpermissive temperature) selecting for chloramphenicol restance (10ug/ml).

One of these chloramphenicol resistant colonies was denoted PL2158

Southern hybridization showed that, in the strain PL2158, the plasmid pPL2002 was integrated into the chromosome by homologous recombination between the plasmid-borne and chromosomal subtilisin 309 genes and thereafter amplified to approx. 4 copies. No evidence of complete pE194 plasmid sequences was detected.

The stability of the chromosomally integrated copies of pPL2002 in strain PL2158 was tested in large scale fermentations (1500 l) without any antibiotic.

After fermentation samples were diluted and plated on TY9 plates and 100 colonies were replicated to TY9 plates containing 10 ug/ml chloramphenicol. 98 of the tested colonies were still resistant to chloramphenicol indicating that the plasmid pPL2002 was still integrated in the chromosome. 20 of these colonies were then tested by Southern hybridisation which showed that the plasmid pPL2002 was still integrated, apparently in the same copy number (approx. 4 copies) in all of the tested colonies.

EXAMPLE 2

Construction of plasmid vectors containing two pUB110 origins of replication

Plasmid pSJ1085 (Fig. 7) was constructed by restricting pDN3060 (containing an origin of replication (+ ori pUB110) and rep gene (rep) from pUB110, and a chloramphenicol resistance gene (cat) from pC194) with BamHI and EcoRI and inserting the following oligonucleotide sequence (prepared by the phosphoamidite method described by Beaucage and Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, on an automatic DNA synthesizer)

AATTCTGCAGATATCAAGATAAGAAAGAACAAGTTCCG
    GACGTCTATAGTTCTATTCTTTCTTGTTCAAGGCCTAG

into the linearized pDN3060 followed by ligation and transformation of B. subtilis DN1885.

Plasmid pSJ1103 (Fig. 9) was constructed by restricting pSJ1085 (Fig. 7) with EcoRI and inserting the entire linearized plasmid into the similarly restricted plasmid pUC19 (Fig. 8) followed by ligation and transformation of E. coli SJ6. The resulting plasmid pSJ1103 contains the plus origin and rep gene from pUB110, the cat gene from pC194, the pUC19 origin of replication (ori pUC19), and the β-lactamase (ampicillin resistance) gene (bla).

Plasmid pSJ1130 (Fig. 10) was derived from pSJ1103 (Fig. 9) by deleting a 1.6 kb NsiI-PstI fragment , essentially resulting in a pUC19 plasmid containing a pUB110 plus-origin and a truncated rep gene (rep'). The plasmid was transformed into E. coli SJ6.

A 1.4 kb HindIII fragment from pDN3060 (Fig. 6) containing the plus-origin followed by the intact rep gene was then inserted into the unique HindIII site of pSJ1130 (Fig. 10), and the ligated plasmid was transformed into E.coli SJ6, resulting in pSJ1136 (Fig. 11). In this experiment, the fragment happened to be inserted into pSJ1136 in two tandem copies. One of these copies was subsequently deleted by digestion of pSJ1136 with NsiI, religation of the 5.1 kb fragment and transformation of E. coli SJ6, resulting in pSJ1137 (Fig. 12) which contains one pUB110 origin next to a truncated rep gene and one pUB110 origin next to an intact rep gene.

The gene encoding kanamycin resistance (kan) was excised from plasmid pPL1484 (Fig. 13) on a 1.4 kb SphI fragment and inserted in each of the two p ssible orientations into the SphI site of pSJ1137 (Fig. 12), f llowed by transformation f

E. coli SJ6, resulting in pSJ1155 (Fig. 14) and pSJ1157 (Fig. 15), respectively . pSJ1157 contained the kan gene in two tandem copies. One copy was excised with BamHI, and the 6.5 kb fragment religated and transformed into E. coli SJ6 to form pSJ1259 (Fig. 16).

Plasmid pSJ1139(Fig. 18) was constructed in the following way : The Bacillus plasmid pDN2904 (Fig. 17), containing a chloramphenicol resistance gene (cat), a kanamycin resistance gene (kan) and the pUB110 plus-origin with the corresponding rep gene was digested with SphI and ligated to pSJ1130 (Fig. 10) which had also been digested with SphI. The resulting plasmid pSJ1139 contains a pUB110 origin associated with a truncated rep gene and a pUB110 origin associated with an intact rep gene.

EXAMPLE 3

Formation of progeny DNA vectors from plasmids containing two pUB110 origins of replication in Bacillus subtilis

Plasmid pSJ1139 (Fig. 18) (prepared from E. coli SJ1139 in a manner known per se) was transformed into B. subtilis DN1885 selecting for kanamycin resistance (10 μg/ml), and plasmid DNA was prepared from several transformants. Agarose gel electrophoresis of these plasmids, whether undigested or digested with a variety of restriction enzymes, showed the presence of two smaller DNA molecules of 5.1 kb and 2.4 kb, respectively, as well as a small amount of the full-length plasmid pSJ1139 of 7.5 kb. The restriction patterns obtained were the expected ones for formation of the two progeny vectors pSJ1139a (Fig. 19) and pSJ1139b (Fig. 20), either by homologous crossing-over between the two rep-sequences on pSJ1139 or by the action of the Rep protein which produces a nick at the pUB110 plus-origin in the plus DNA strand which is then displaced and recircularized as described in A. Gruss and S. D. Erlich, op. cit. (both these mechanisms could lead to the same two progeny vectors.

These vectors were further analyzed by retransformation into B. subtilis strain DN1885, and plating on LB plates containing either 10 μg/ml kanamycin or 6 μg/ml chloramphenicol, followed by replica plating of each plate onto a new plate containing the other antibiotic. Vectors were then isolated from each type of transformant and analyzed by agarose gel electrophoresis with the following results:

Transformants resistant to both chloramphenicol and kanamycin contain all three vector species(pSJ1139 of 7.5 kb, pSJ1139a of 2.4 kb and pSJ1139b of 5.1 kb). Transformants resistant to chloramphenicol and sensitive to kanamycin only contain pSJ1139b. Transformants resistant to kanamycin but sensitive to chloramphenicol were not obtained. The small progeny vector pSJ1139a of 2.4 kb is thus not able to replicate autonomously in B. subtilis.

EXAMPLE 4

Stable integration of a non-replicative DNA molecule in the B. subtilis chromosome

Construction of a B. subtilis strain containing one chromosomal copy of a cyclodextrin glycosyl transferase (CGTase) gene

The CGTase gene (CGT) was excised from plasmid pPL1878 (Fig. 22) on a 2.5 kb BamHI-SphI fragment and ligated to the BamHI-SphI digested plasmid pDN3020 (Fig. 21) to form plasmid pPL1896 (Fig. 23). pDN3020 is a derivative of pDN1313 (Diderichsen, 1986), constructed by inserting a synthetic SphI-containing oligonucleotide linker (prepared as described in Example 1 above) into the EcoRI site of plasmid pDN1380 (Diderichsen and Christiansen, 1988) resulting in plasmid pDN1620. The promoter region from a maltogenic amylase from B. stear th rmophilus (PamyM), present on pDN1620 (B.

Diderichsen and L. Christiansen, op. cit.) was then transferred to SphI-BamHI digested pUC19 on an approximately 200 bp BamHI-SphI fragment resulting in plasmid pDN2977. The promoter region was excised from pDN2977 on an approximately 200 bp BglII-SacI fragment which was inserted into the polylinker region of pDN1313, thereby generating plasmid pDN3020. The CGTase gene on pPL1896 is flanked by two fragments of B. subtilis chromosomal DNA indicated as dal and dfs in Fig. 23. dal is the gene encoding D,L-alanine racemase of B. subtilis (Diderichsen 1986).

Plasmid pPL1896 was transformed into B. subtilis strain DN1686. When selecting solely for Dal^+ transformants, several strains were obtained that were chloramphenicol sensitive, $CGTase^+$. They were formed by a double homologous crossing-over between pPL1896 and the DN1686 chromosome, as described in Diderichsen, 1986. One such strain is PL1897, containing a chromosomally integrated copy of the CGTase gene.

Construction of an integration vector containing the CGTase gene

The CGTase gene was excised from pPL1878 (Fig. 22) on a 2.5 kb BamHI-NotI fragment. An expression vector was constructed by inserting a 0.6 kb SphI-PstI fragment containing the promoter region of the alpha-amylase gene cloned from an amylase-overproducing derivative of B. licheniformis ATCC9789 obtained by conventional mutagenesis procedures into a pUB110 derived vector containing the pUB110 origin and the gene encoding kanamycin resistance. The CGTase gene (cgt) was inserted downstream of this promoter between the BamHI and NotI sites, resulting in pSJ993 (Fig. 24).

A 4 kb BglII fragment from pSJ993 was inserted into the BglII site of pSJ1155 (described in Example 1 above, Fig. 14), the resulting plasmid was transformed into E. coli strain SJ6, and ampicillin-resistant, CGTase-producing transformants of E. coli SJ6 were isolated by plating transformants on LB

plates containing 100 μg/ml ampicillin and 0.5% soluble starch, screening for the formation of a clear halo around the colonies after staining the plates with iodine vapour. A transformant harbouring the plasmid pSJ1163 (Fig. 25) in which the kanamycin resistance gene had been regenerated was kept for further experiments.

Formation of progeny vectors from pSJ1163 in <u>B. subtilis</u> strains DN1885 and PL1897

pSJ1163 (Fig. 25) was transformed into DN1885 and vector DNA was prepared from kanamycin-resistant transformants and analyzed by agarose gel electrophoresis. This showed traces of a 10.5 kb plasmid molecule, corresponding to pSJ1163, and two progeny vector molecules pSJ1163b of 4.1 kb (Fig. 27) and pSJ1163a of 6.4 kb (Fig. 26) in approximately equal and in far larger amounts, respectively, corresponding to progeny vectors derived either by homologous recombination between the two <u>rep</u>-sequences of pSJ1163 or by the action of the Rep protein at each plus origin in rolling circle replication as described above. The formation of progeny vectors as described above was also observed when pSJ1163 was transformed into PL1897, and two such transformants were kept for further experiments as strains SJ1168 and SJ1170.

Isolation of integrants containing non-replicative DNA molecules

Strains SJ1168 and SJ1170 were inoculated into 10 ml TY medium containing 5 μg/ml kanamycin and incubated overnight at 37°C. 100 μl of each culture were then inoculated into fresh TY medium and the incubation was repeated. After four such cycles of incubation overnight, plasmid DNA was prepared from the two cultures and analyzed by agarose gel electrophoresis. No plasmid molecules were observed. When the plasmid preparation was used to transform <u>E. coli</u> selecting for ampicillin resistance, no transformants were obtained, indicating that neither the original 10.5 kb pSJ1163 nor th

4.1 kb progeny vector molecule pSJ1163b were present. The kanamycin-resistant, plasmid-free strains were kept for further experiments as SJ1223 and SJ1237.

Amplification of integrated DNA

By selecting for growth in TY medium containing gradually increasing concentrations of kanamycin, strains were isolated that were able to grow in 20, 50, 100, 200, 400, 600, 800, 1000, 1200, and 1400 µg/ml kanamycin. In chromosomal DNA from strains resistant to above about 400 ug/ml kanamycin, digestions with NheI or NotI revealed a DNA band of the size expected from digestion of the 6.4 kb progeny vector pSJ1163a with these enzymes. This band did not appear in digests of DNA prepared from strains with a lesser degree of kanamycin resistance. A conservative estimate would be that at least 5-10 copies of the integrated DNA were present when this band appeared.

Stability of integrated DNA

Strains resistant to 400 µg/ml kanamycin were grown for one week at 37 °C in shake flasks containing BPX medium without any added kanamycin. They were then plated onto LB plates and subsequently replica plated onto plates containing 10 µg/ml kanamycin. Of about 100 colonies, all were kanamycin resistant, indicating the stable inheritance of the kanamycin resistance gene present on the integrated DNA in the abscence of selection pressure.

Stability of a plasmid-borne CGTase gene in B. subtilis

Plasmid pPl1892 is essentially identical to pSJ993 (Fig. 24), the only difference being that a different polylinker region is present downstream of the CGTase gene. This plasmid was introduced into DN1885 and the resulting strain SJ984 was grown for one week at 37°C in shake flasks containing BPX medium without any added kanamycin.

Plating on kanamycin-containing plates (10 µg/ml) gave a 10-fold lower cell count than plates without kanamycin, indicating that 90% of the cells had lost their plasmid. This was also reflected by the finding that less than 10% of the colonies on plates without kamamycin produced CGTase.

EXAMPLE 5

Formation of progeny vectors from pSJ1156 in B. licheniformis ATCC 9789.

Plasmid pSJ1156 is identical to pSJ1157 shown in Fig. 15. pSJ1156 was introduced into B. licheniformis ATCC 9789 by protoplast transformation, selecting for kanamycin resistance, resulting in strain SJ1199. Analysis of the plasmid content of SJ1199 by restriction enzyme digestion and agarose gel electrophoresis revealed the presence of two plasmid molecules. One was identical to pSJ1259 (Fig. 16) and most likely formed by deletion of one copy of the kan gene by homologous recombination. The other corresponded to pSJ1259a (Fig. 28), one of the two progeny molecules that could be formed either by homologous recombination between the two rep sequences of pSJ1259 or by the action of the Rep protein at each plus origin in rolling circle replication as described above.

EXAMPLE 6

Stable integration of a non-replicative DNA molecule in the B. licheniformis ATCC 9789 chromosome

Construction of integration vector

Plasmid pSJ1260 is identical to pSJ1259 shown in Fig. 16. Chromosomal DNA from B. licheniformis ATCC 9789 was digested with PstI + BamHI and fragments between 2 and 4 kb were isolated from an agarose gel. These fragments were ligated

into pSJ1260 digested with PstI + BamHI, and transformed into E. coli SJ6 selecting ampicillin resistance. One transformant obtained contained an insert of 2.1 kb and the plasmid was denoted pSJ1555 (Fig. 29). E. coli SJ6 containing pSJ1555 was deposited at the National Collection of Industrial and Marine Bacteria Ltd, 23 St. Machar Drive, Aberdeen, AB2 1RY, Scotland, UK on December 12, 1990 in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, with the accession number NCIMB 40346. This plasmid has the capacity to form the two progeny molecules pSJ1555a (Fig. 30) and pSJ1555b (Fig. 31).

Isolation of B. licheniformis integrant containing a non-replicative DNA molecule.

pSJ1555 was introduced into B. licheniformis ATCC 9789 by protoplast transformation, selecting for kanamycin resistance. One regenerated, kanamycin-resistant transformant (SJ1613) was plasmid-free as seen by gel electrophoresis of a plasmid-preparation from that transformant, and the plasmid preparation was unable to transform B. subtilis to kanamycin resistance. This result indicates that the non-replicative progeny molecule pSJ1555a had formed and had been integrated into the ATCC 9789 chromosome.

Amplification and stability of integrated DNA

Strain SJ1613 was grown in successive 10 ml TY cultures containing kanamycin at 10, 20, 50, 100, 200, 400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000 and 5000 ug/ml, and strains growing at each of these different concentrations were kept for further study. Strains resistant to 20, 200 and 1500 ug/ml kanamycin were further analyzed. Chromosomal DNA from the two latter strains revealed upon digestion with BamHI a distinct band of 4.5 kb, absent from DNA of the first strain, as expected for strains containing multiple copies of pSJ1555a integrated in the chromosome.

All strains were grown in BPX shake flasks at 37 °C for 7 days without kanamycin, and then streaked on LB plates. Replica plating from LB plates to kanamycin plates (10 ug/ml) revealed no kanamycin sensitive colonies. Colony counts on plates with and without kanamycin (10 ug/ml) were obtained for the three strains resistant to 20, 200 and 1500 ug/ml kanamycin, and were in all cases 10^{10} ml^{-1}, indicating stability of the integrated _kan_ gene.

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International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 32, line 4 of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

NATIONAL COLLECTIONS OF INDUSTRIAL & MARINE BACTERIA

Address of depositary institution (including postal code and country) [4]

23 St. Machar Drive, Aberdeen, AB2 1RY, Scotland, UK

Date of deposit [5]	Accession Number [6]
12 December 1990	NCIMB 40346

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28 (4) EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

Ulla Gertsen

(Authorized Officer)

Ulla Gertsen
Head Clerk

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

CLAIMS

1. A bacterial cell which in its genome carries an integrated non-replicative DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of the cell, and (3) an origin of replication, said DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication.

2. A cell according to claim 1, wherein the DNA construct has been deleted of the gene encoding the replication factor.

3. A cell according to claim 1, wherein the gene encoding the replication factor has been modified so as to encode an inactive replication factor, or modified so that no replication factor is expressed from the gene.

4. A cell according to claim 3, wherein said gene has been modified by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by deletion or other modification of transcriptional or translational start or stop signals.

5. A cell according to any of claims 1-4, wherein the DNA construct comprises a DNA sequence of interest, a DNA sequence which is homologous to a region of the genome of the cell, and a plus origin of replication from a single-strand DNA plasmid, the DNA construct lacking a functional rep gene cognate to the plus origin of replication.

6. A cell according to any of claims 1-5, wherein the DNA construct additionally comprises a selectable marker.

7. A cell according to any of claims 1-5, wherein the DNA sequence of interest encodes a polypeptide of interest.

8. A cell according to any of claims 1-7, which is a cell of a gram-positive bacterium.

9. A cell according to claim 8, wherein the gram-positive bacterium is a strain belonging to the genus Bacillus or Streptomyces.

10. A cell according to claim 9, wherein the bacterium is a strain of Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus subtilis or Streptomyces lividans.

11. A DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the DNA construct, and (3) an origin of replication, said DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication.

12. A DNA construct according to claim 11, which has been deleted of the gene encoding the replication factor.

13. A DNA construct according to claim 11, wherein the gene encoding the replication factor has been modified so as to encode an inactive replication factor, or modified so that no replication factor is expressed from the gene.

14. A DNA construct according to claim 13, wherein said gene has been modified by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by deletion or other modification of transcriptional or translational start or stop signals.

15. A DNA construct according to any of claims 11-14, comprising a DNA sequ nce of interest, a DNA sequence which is homolog us with a region f the genome of a cell intended

for introduction of the DNA construct, and a plus origin of replication from a single-strand DNA plasmid, the DNA construct lacking a functional rep gene cognate to the plus origin.

16. A DNA construct according to claim 11 which additionally comprises a selectable marker.

17. A recombinant DNA vector comprising a DNA construct according to any of claims 11-16.

18. A parental plasmid vector which comprises (i) a first origin of replication; (ii) one or more functional genes encoding the replicational factor(s) required for replication from said first origin of replication; (iii) a second origin of replication in the same orientation as the first origin of replication; (iv) a DNA sequence of interest, and (v) a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the vector, said parental vector lacking a functional gene encoding a replication factor required for replication from the second origin of replication in the region between the second and the first origin of replication (in the above-mentioned order).

19. A plasmid vector according to claim 18, wherein the second origin of replication is derived from the same plasmid as the first origin of replication.

20. A plasmid vector according to claim 18, which has been deleted of the gene encoding the replication factor associated with the second origin of replication.

21. A plasmid vector according to claim 18, wherein the gene encoding the replication factor associated with the second origin of replication has been modified.

22. A plasmid vector according to claim 21, wherein the gene has been modified by deletion, insertion or substitution of

one or more nucleotides of the DNA sequence of the gene, or by deletion of transcriptional or translational start or stop signals.

23. A parental plasmid vector which comprises (i) a first plus origin from a single-stranded DNA plasmid; (ii) a functional rep gene cognate to the first plus origin; (iii) , a second plus origin from a single-stranded DNA plasmid in the same orientation as the first plus origin; (iv) a DNA sequence of interest, and (v) a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the plasmid vector, said parental vector lacking a functional rep gene cognate to the second plus origin in the region between the second and the first plus origin of replication (in the above-mentioned orientation).

24. A plasmid vector according to claim 23, wherein the second plus origin of replication is derived from the same single-strand DNA plasmid as the first plus origin of replication.

25. A plasmid vector according to any of claims 18-24, which further comprises a selectable marker.

26. A bacterial cell which comprises a first DNA vector comprising a first origin of replication and one or more functional gene(s) encoding the factor(s) required for plasmid replication from said first origin of replication, and a second DNA vector comprising a second origin of replication but lacking afunctional gene encoding a factor required for plasmid replication from said second origin of replication, as well as comprising a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of the cell.

27. A cell according to claim 26, wherein the second origin of replication is derived from th same plasmid as the first origin f replication.

28. A cell according to claim 26, wherein the second DNA vector has been deleted of the gene encoding the replication factor cognate to the second origin of replication.

29. A cell according to claim 26, wherein the gene encoding the replication factor cognate to the second origin of replication has been modified.

30. A cell according to claim 29, wherein the gene has been modified by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by deletion or other modification of transcriptional or translational start or stop signals.

31. A cell according to claim 26, wherein the first DNA vector further comprises a functional gene encoding the replication factor cognate to the second origin of replication.

32. A cell according to claim 26, wherein the second DNA vector further comprises a selectable marker.

33. A cell according to any of claims 26-32, which comprises a first DNA vector comprising a first plus origin of replication from a single-stranded DNA plasmid and a functional rep gene, and a second DNA vector comprising a second plus origin of replication from a single-stranded DNA plasmid but lacking a functional rep gene cognate to the second plus origin of replication, as well as a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of said cell.

34. A cell according to claim 33, wherein the second plus origin of replication is derived from the same single-stranded DNA plasmid as the first plus origin of replication.

35. A cell according to any of claims 26-34, which is a cell of a gram-positive bacterium.

36. A cell according to claim 35, wherein the gram-positive bacterium is a strain belonging to the genus Bacillus or Streptomyces.

37. A cell according to claim 36, wherein the bacterium is a strain of Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus subtilis or Streptomyces lividans.

38. A method of producing a bacterial cell according to any of claims 1-10, the method comprising transforming bacterial cells with a parental plasmid vector according to any of claims 18-25, and culturing the transformed cells under selective conditions, replication of the plasmid vector giving rise to the formation of a first progeny vector comprising a first origin of replication and one or more functional gene(s) encoding the factor(s) required for plasmid replication from said first origin of replication, and a second progeny vector comprising a second origin of replication but lacking a functional gene encoding a factor required for plasmid replication from said second origin of replication, as well as comprising a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of the cell, continued culturing of the transformed cells under selective conditions resulting in the integration of said second progeny vector into the bacterial genome by homologous recombination and loss of the first progeny vector as well as the parental plasmid from the cells.

39. A method according to claim 38, wherein the parental plasmid vector is one which is unable to replicate at increased temperatur s which yet permit growth of the host cell, and wherein the bacterial cells are initially cultured

at a temperature permitting plasmid replication and subsequently, after integration of the second progeny vector into the bacterial genome, cultured at a temperature which does not permit plasmid replication so that the first progeny vector as well as the parental vector are lost from the cells.

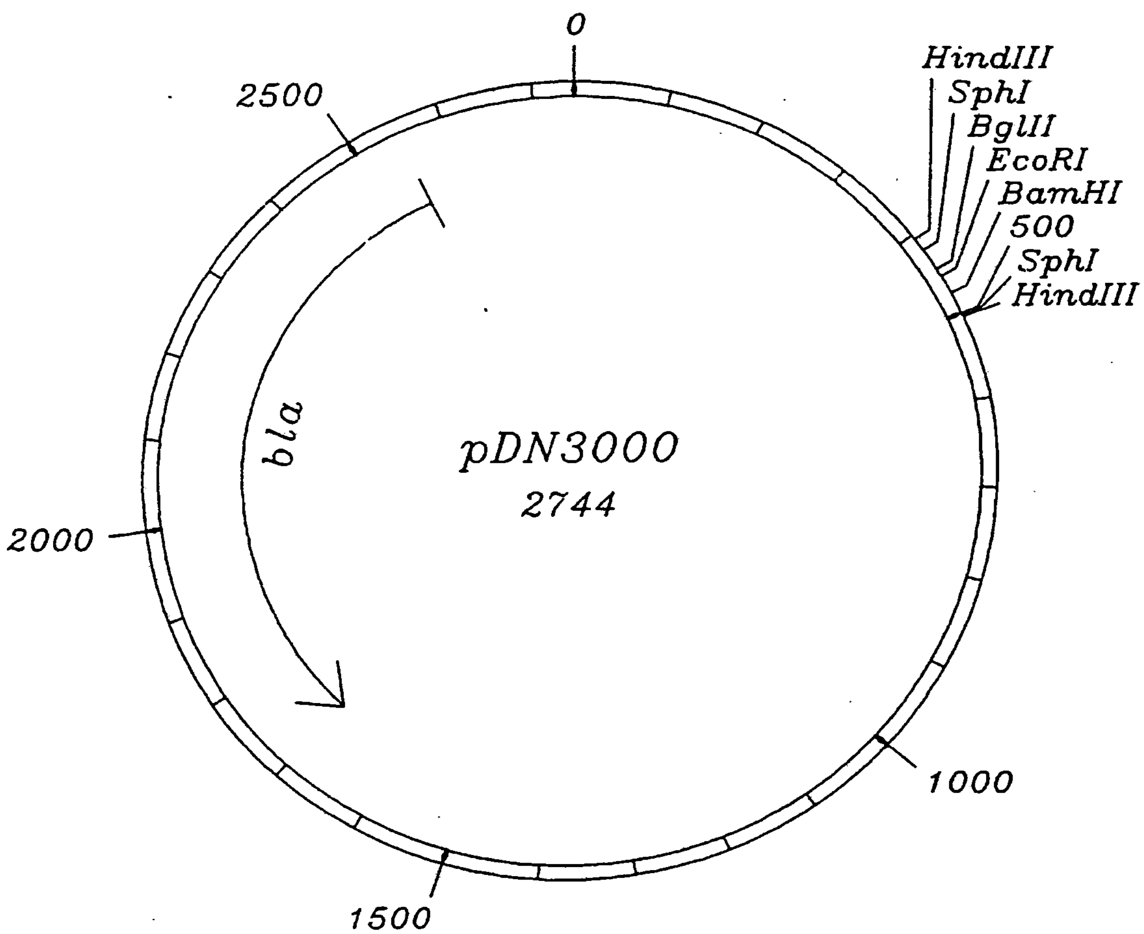
40. A method of producing a bacterial cell according to any of claims 1-10, the method comprising transforming bacterial cells with a first DNA vector comprising a first origin of replication and one or more functional gene(s) encoding the factor(s) required for plasmid replication from said first origin of replication, and with a second DNA vector comprising a second origin of replication but lacking a functional gene encoding a factor required for plasmid replication from the second origin of replication, as well as comprising a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of the cell, and culturing the resulting cells under selective conditions leading to integration of said second DNA vector into the bacterial genome by homologous recombination and loss of the first DNA vector.

41. A method according to claim 40, wherein the first DNA vector is one which is unable to replicate at increased temperatures which yet permit growth of the host cells, and wherein the bacterial cells are initially cultured at a temperature permitting plasmid replication and subsequently, after integration of the second DNA vector into the bacterial genome, cultured at a temperature which does not permit plasmid replication so that the first DNA vector is lost from the cells.

42. A method according to claim 40, wherein the first DNA vector further comprises one or more functional gene(s) encoding the replication factor(s) required for replication from the second origin of replication.

43. A process for producing a polypeptide of interest, comprising culturing a bacterial cell according to any of claims 1-10 containing an integrated DNA sequence which codes for said polypeptide under conditions conducive to the production of the polypeptide and recovering the resulting polypeptide from the culture.

44. A process according to claim 43, wherein the polypeptide is an enzyme, e.g. a protease, amylase or lipase.


0
HindIII
SphI
BglII
EcoRI
BamHI
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SphI
HindIII
2500
bla
pDN3000
2744
2000
1000
1500

Fig. 1

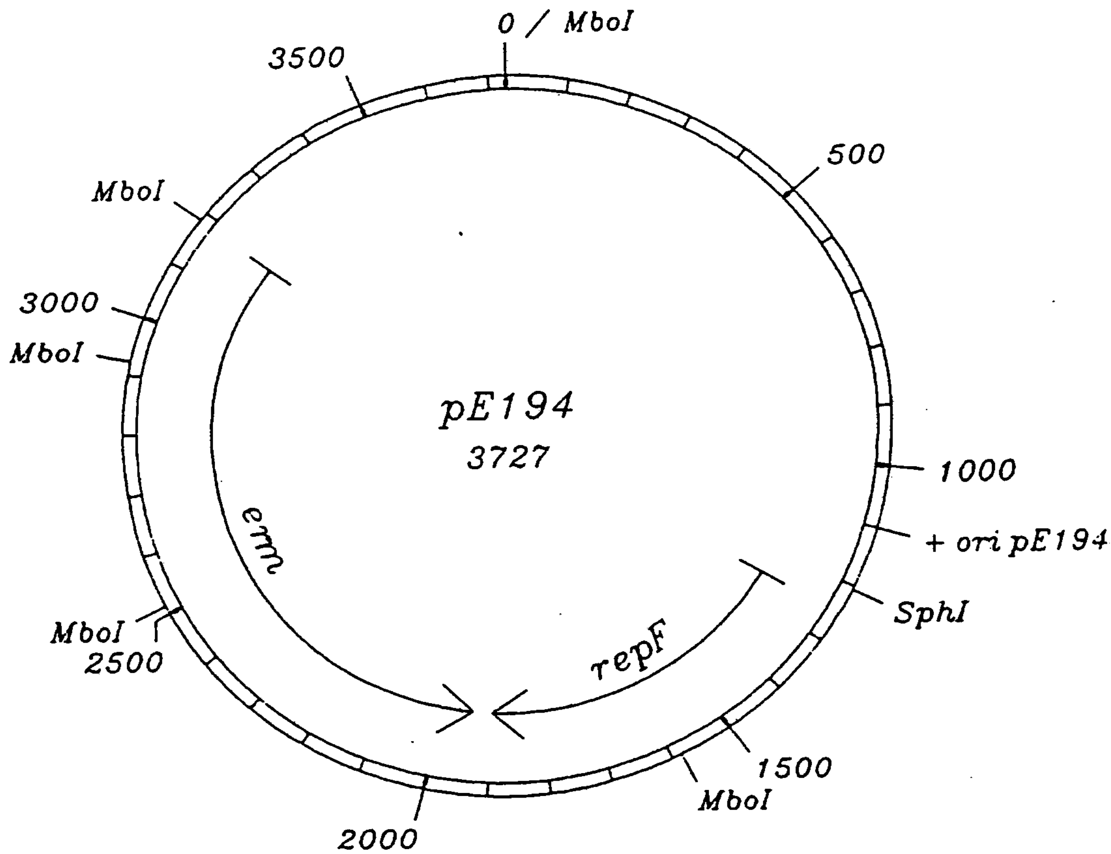
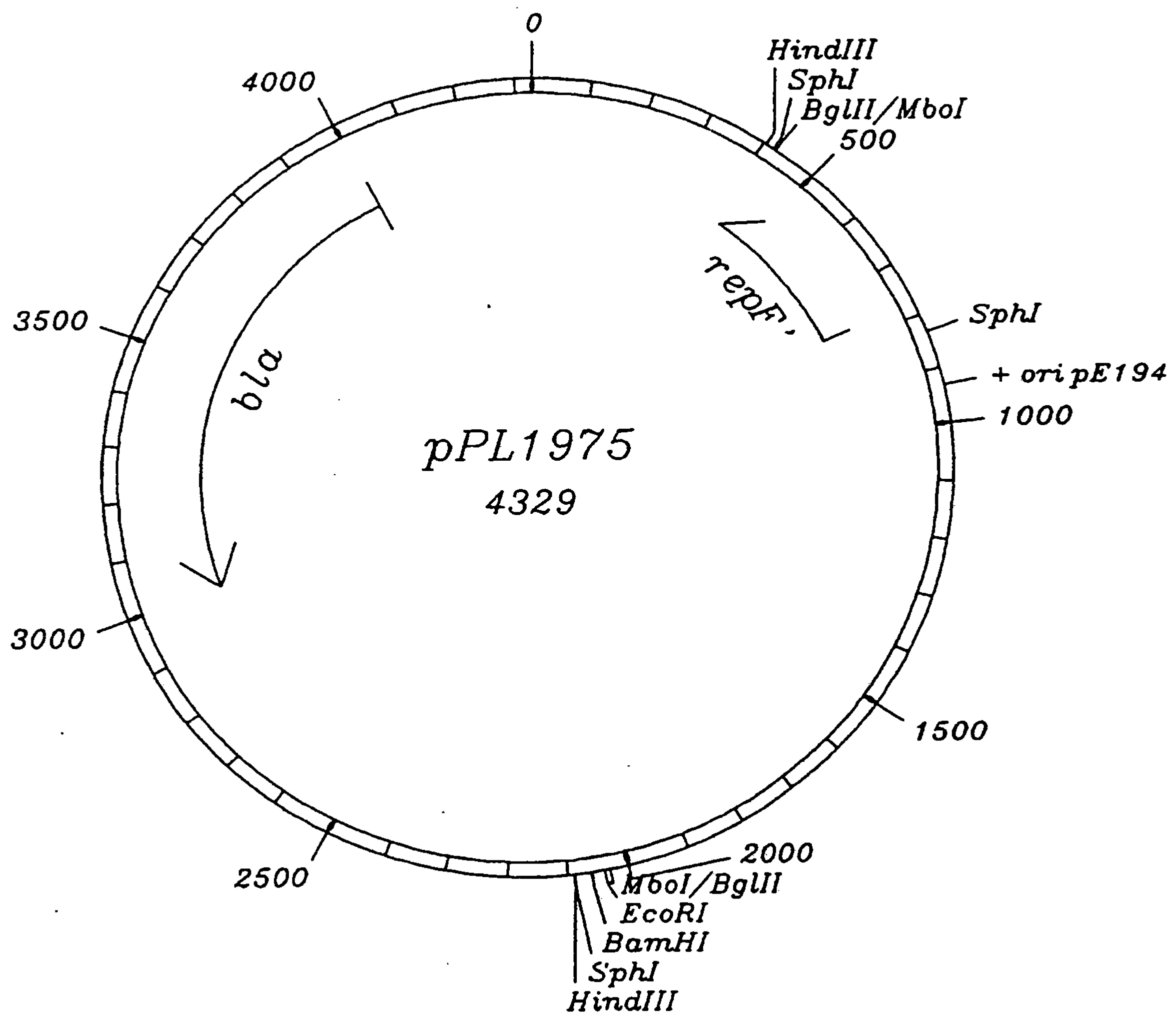

0 / MboI
3500
500
MboI
3000
MboI
pE194
3727
1000
+ ori pE194
erm
SphI
repF
MboI
2500
1500
MboI
2000

Fig. 2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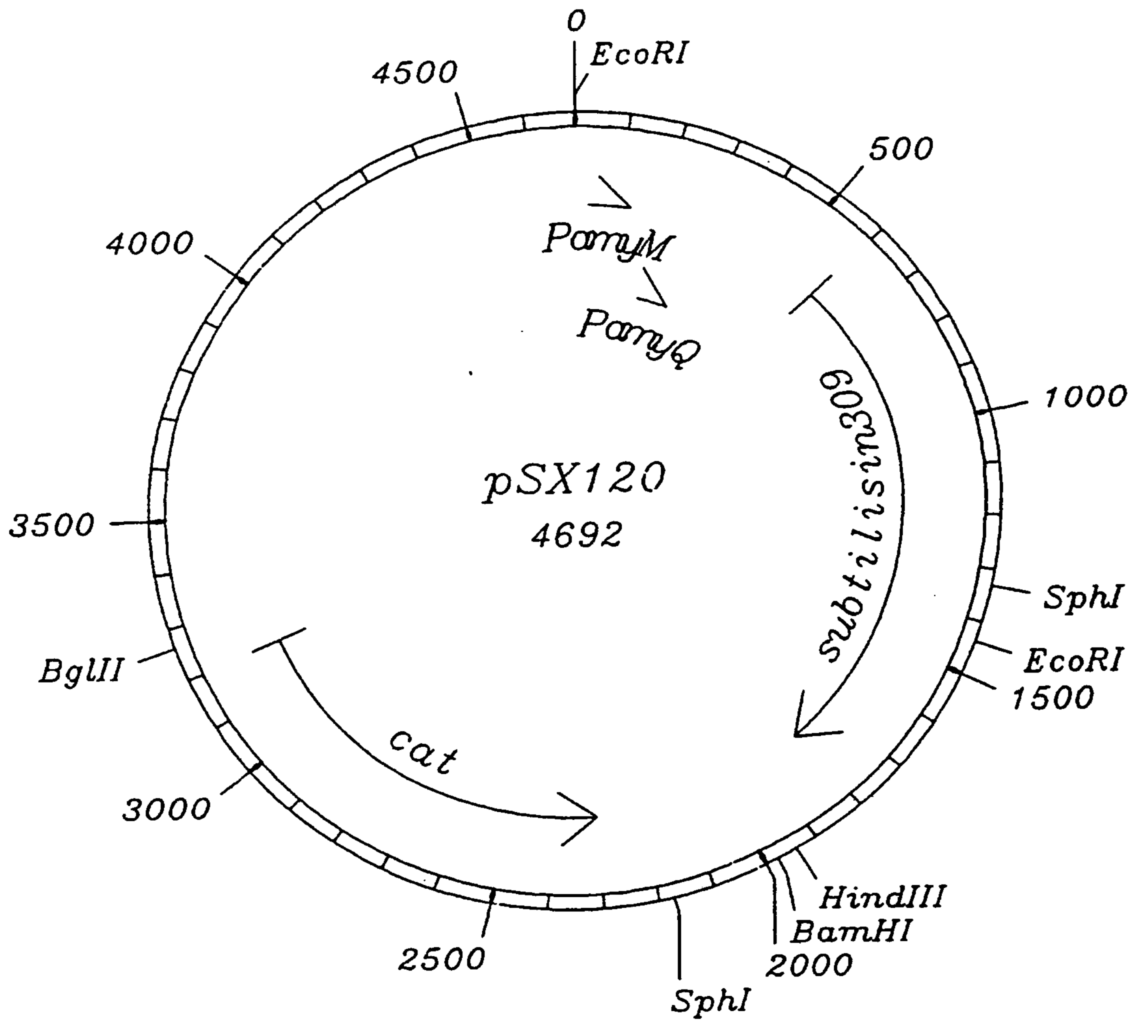
SphI
BglII/MboI
500
SphI
+ ori pE194
1000
1500
2000
MboI/BglII
EcoRI
BamHI
SphI
HindIII
2500
3000
3500
4000
repF
bla
pPL1975
4329

Fig. 3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4500
500
4000
PamyM
PamyQ
subtilisin309
1000
pSX120
4692
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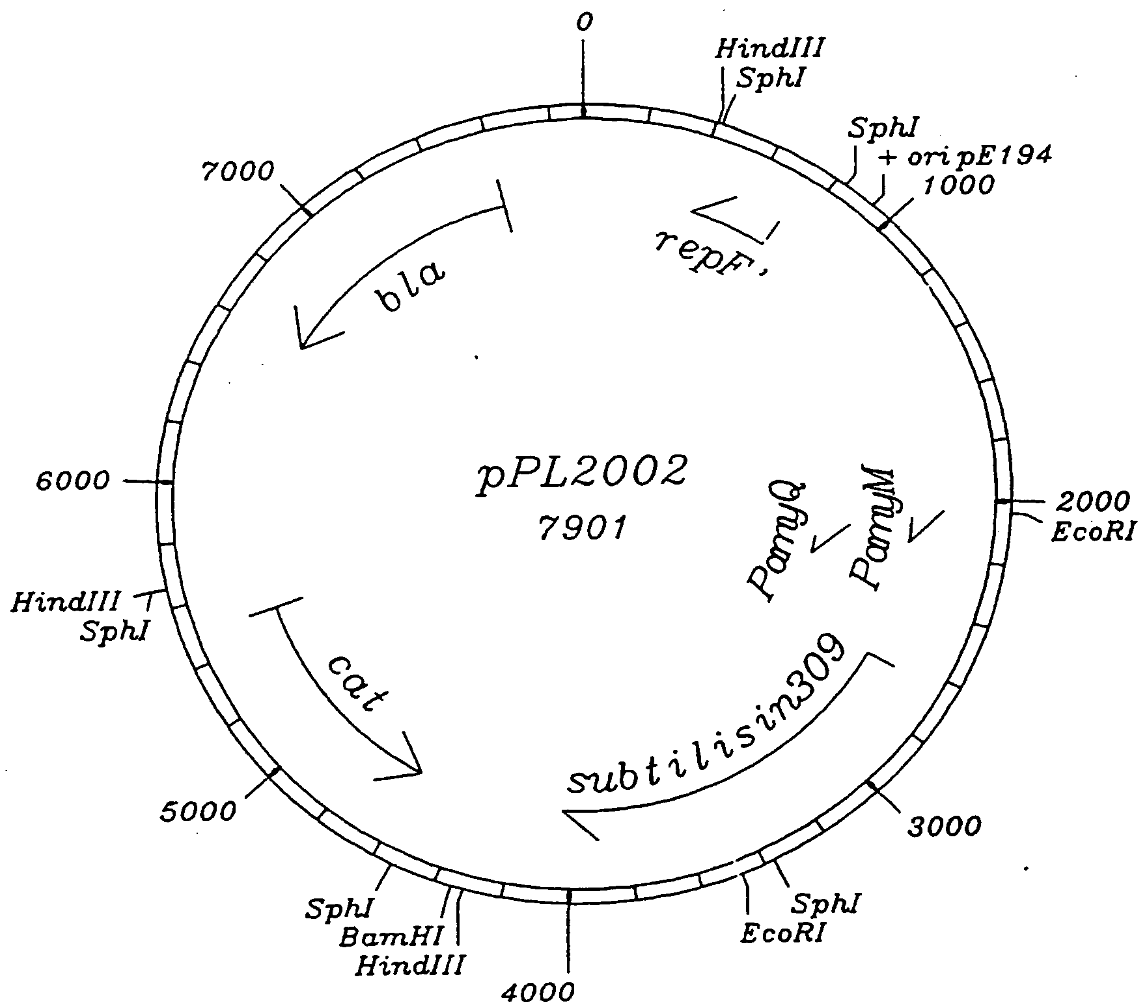
SphI
EcoRI
BglII
1500
cat
3000
HindIII
BamHI
2500
2000
SphI

Fig. 4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HindIII
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SphI
+ ori pE194
1000
7000
repF'
bla
pPL2002
7901
6000
PamyQ
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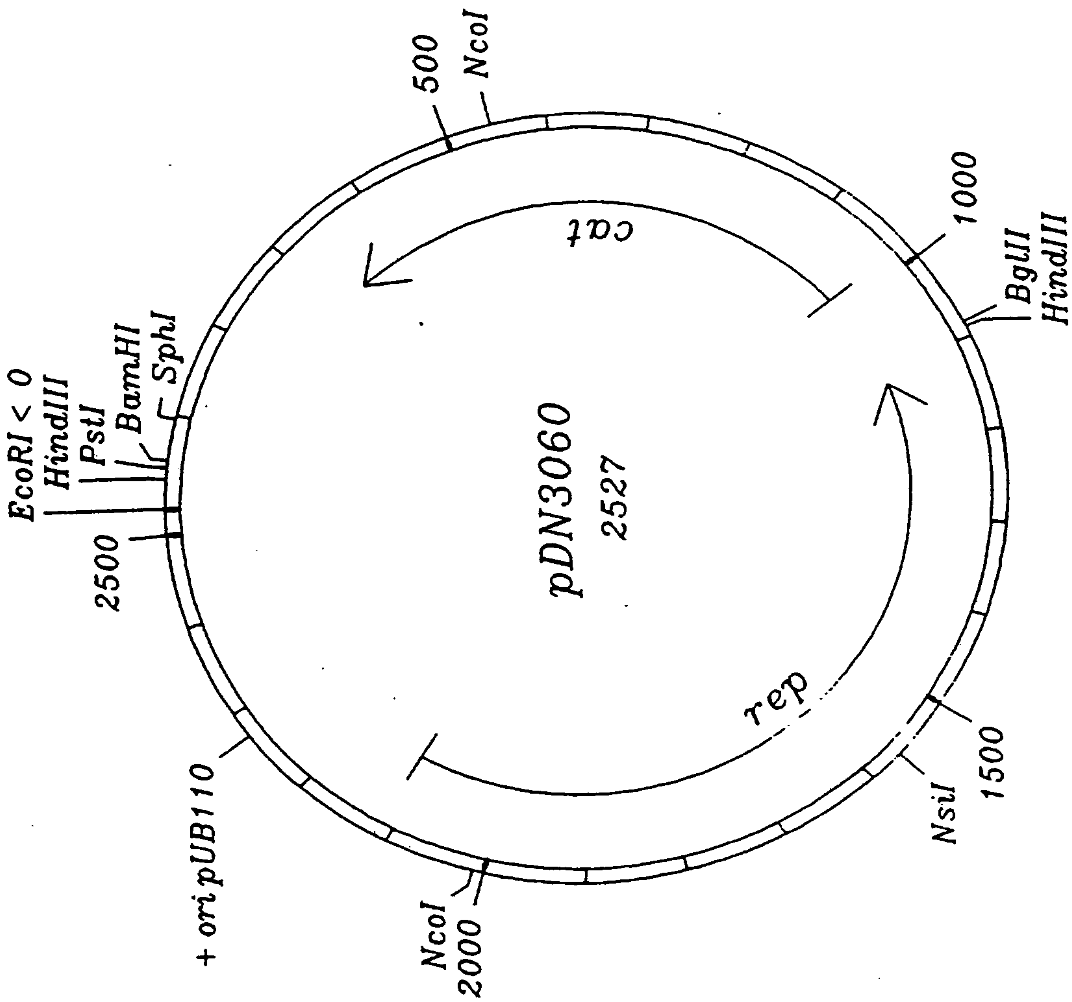
2000
EcoRI
HindIII
SphI
cat
subtilisin309
5000
3000
SphI
BamHI
HindIII
4000
SphI
EcoRI

Fig. 5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2527
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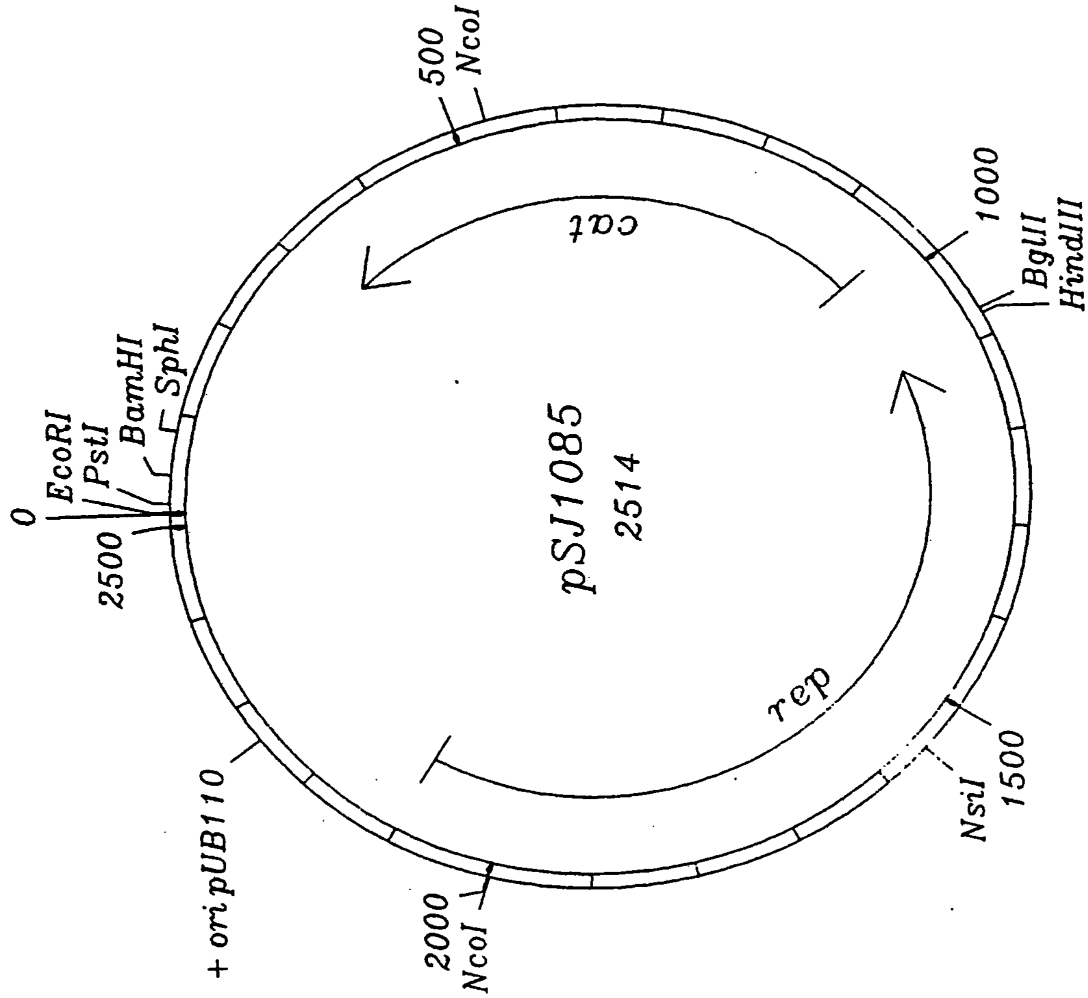
NcoI
1000
BglII
HindIII
1500
NsiI
NcoI
2000
+ ori pUB110
2500
EcoRI < 0
HindIII
PstI
BamHI
SphI
cat
rep

Fig. 6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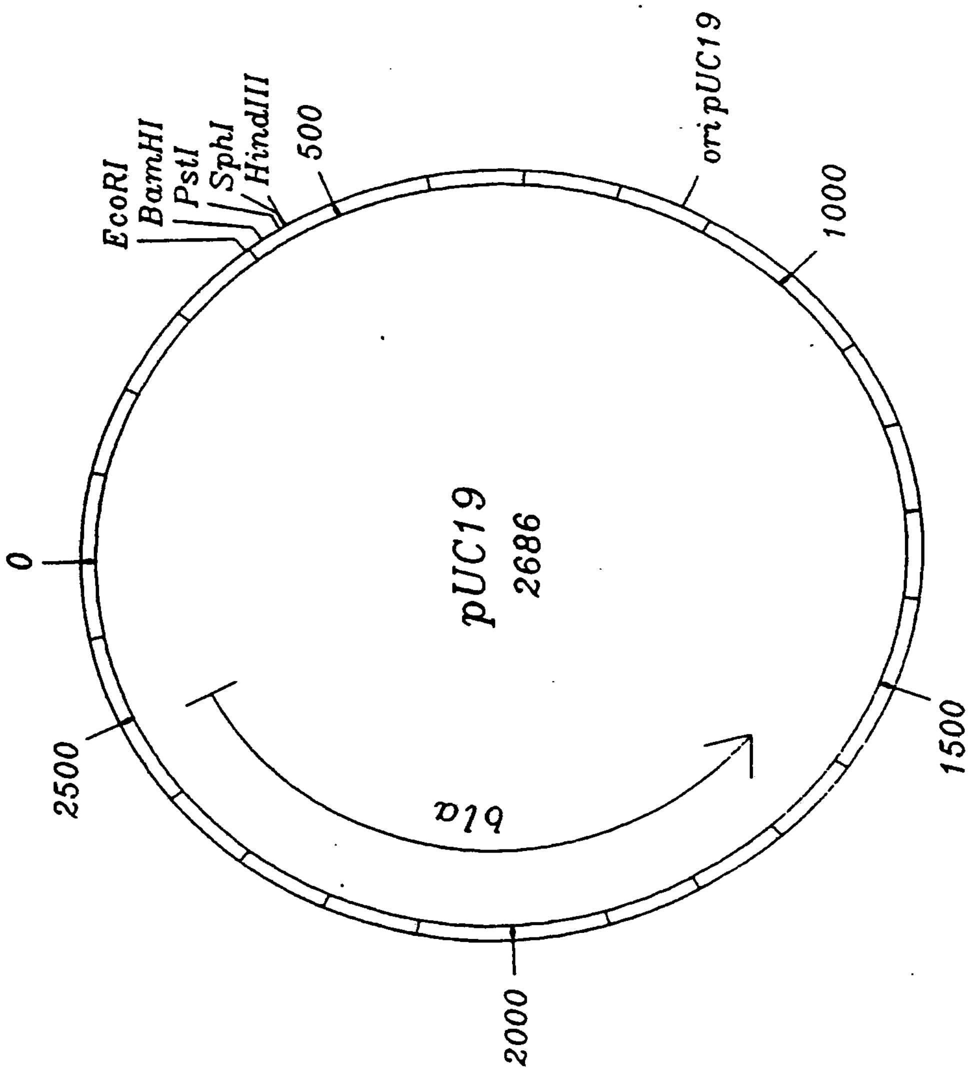
500
NcoI
1000
BglII
HindIII
1500
NsiI
2000
NcoI
+ ori pUB110
2500
0
EcoRI
PstI
BamHI
SphI
cat
rep

Fig. 7


EcoRI
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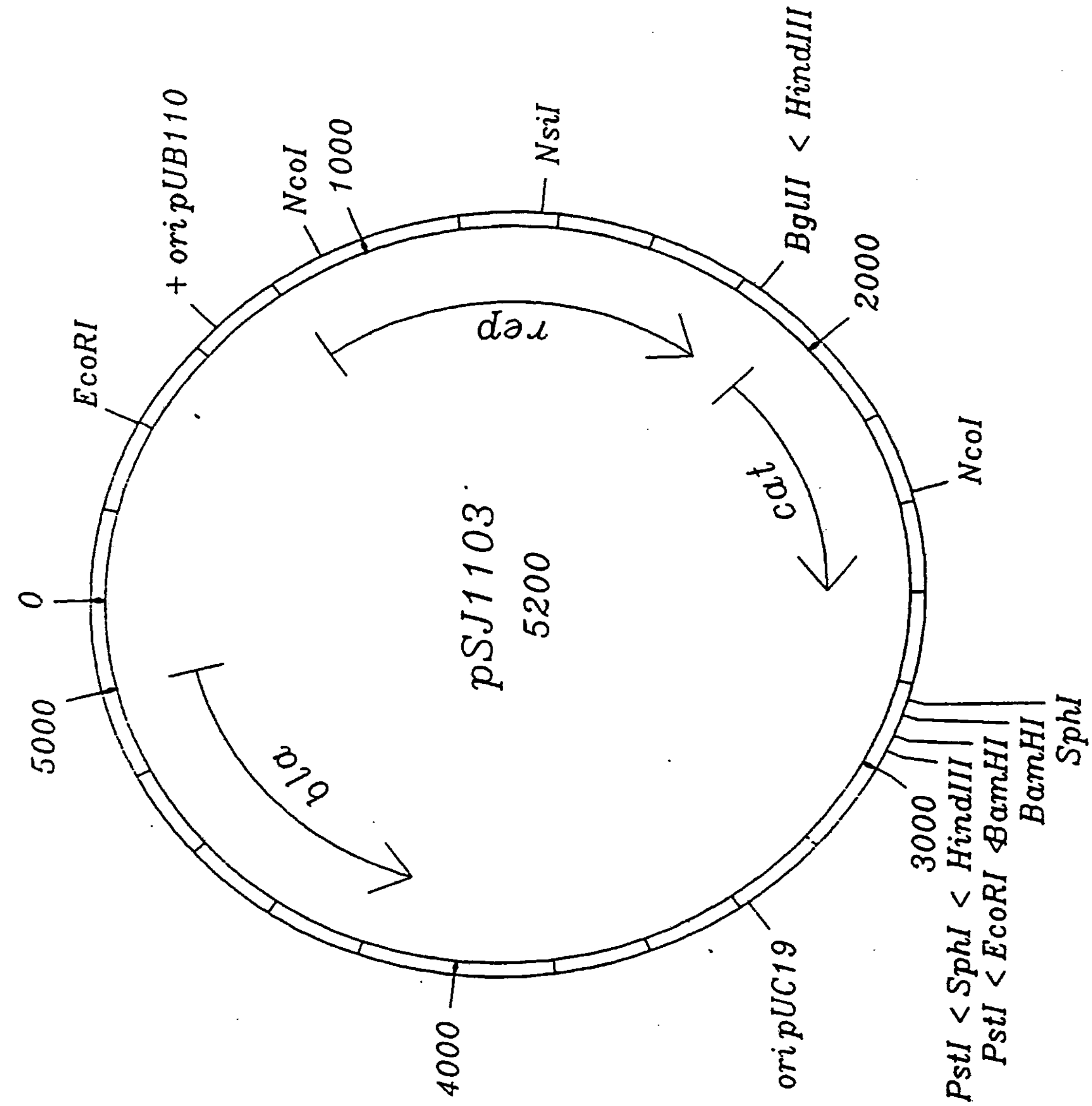
SphI
HindIII
500
oripUC19
1000
1500
2000
2500
0
pUC19
2686
bla

Fig. 8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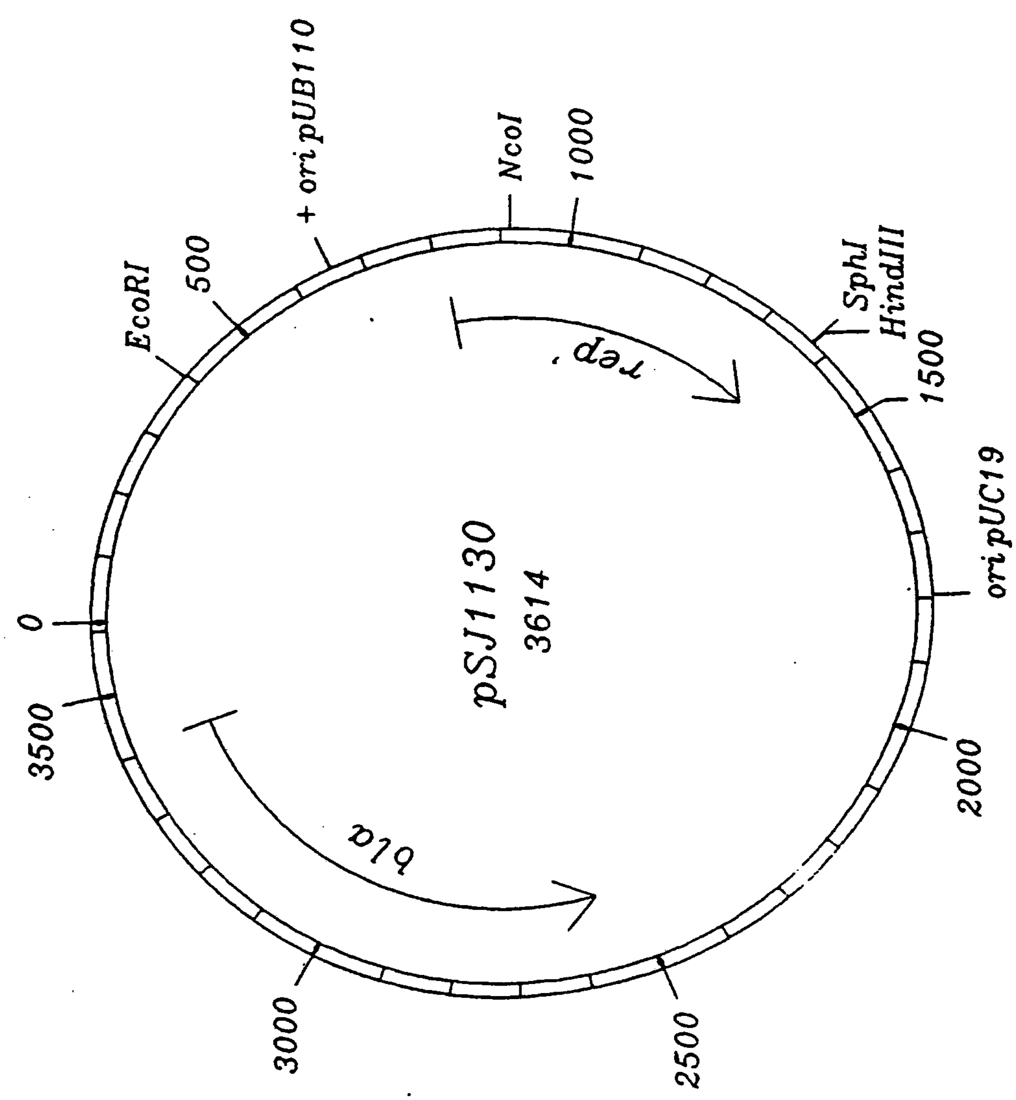
5200
0
1000
2000
3000
4000
5000
EcoRI
+ ori pUB110
NcoI
NsiI
BglII < HindIII
NcoI
SphI
BamHI
HindIII
PstI < SphI < HindIII
PstI < EcoRI < BamHI
ori pUC19
rep
cat
bla

Fig. 9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3614
+ ori pUB110
NcoI
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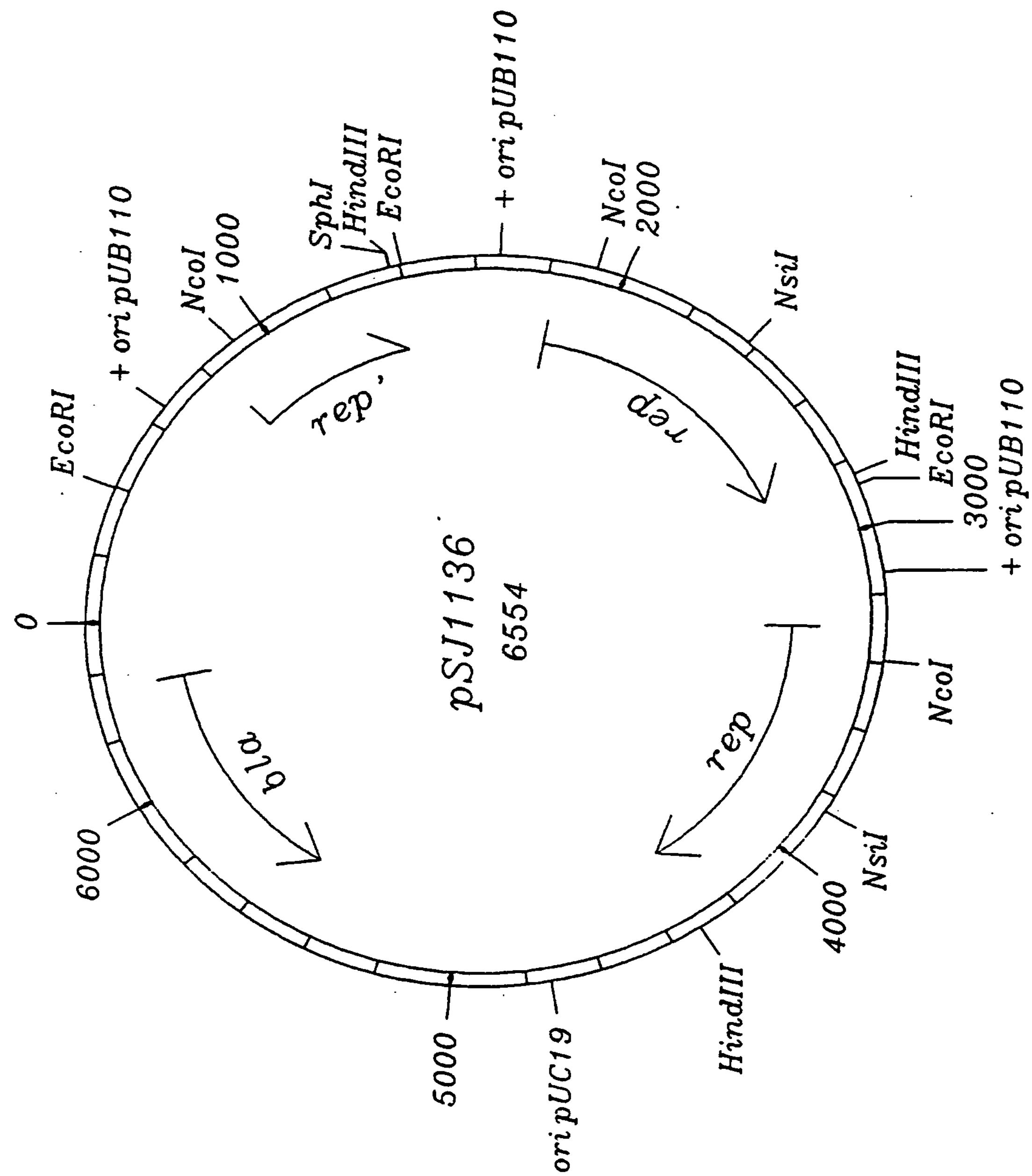
SphI
HindIII
1500
ori pUC19
2000
2500
3000
3500
0
EcoRI
500
rep
bla

Fig. 10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+ ori pUB110
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1000
SphI
HindIII
EcoRI
+ ori pUB110
NcoI
2000
NsiI
HindIII
EcoRI
3000
+ ori pUB110
NcoI
NsiI
4000
HindIII
ori pUC19
5000
6000
rep'
rep
rep
bla

Fig. 11

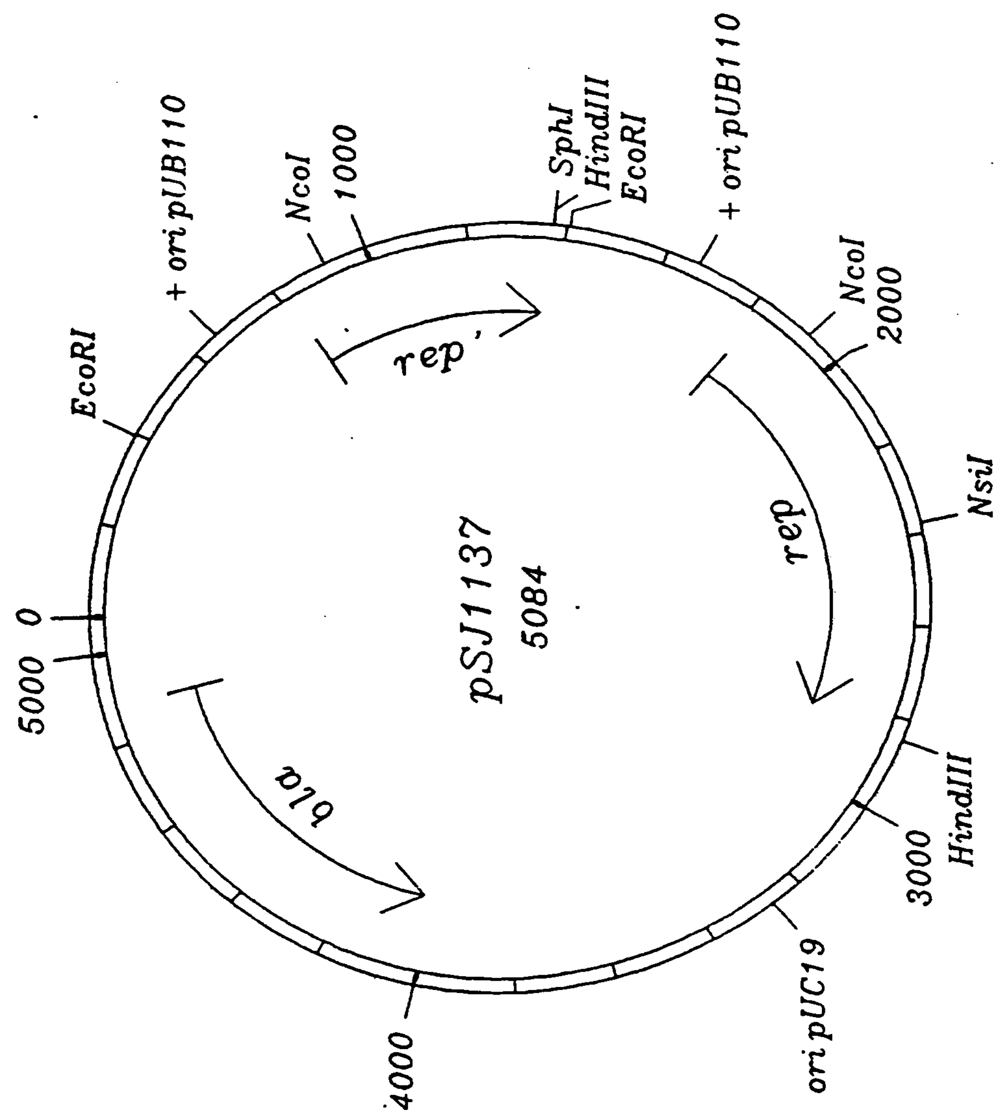
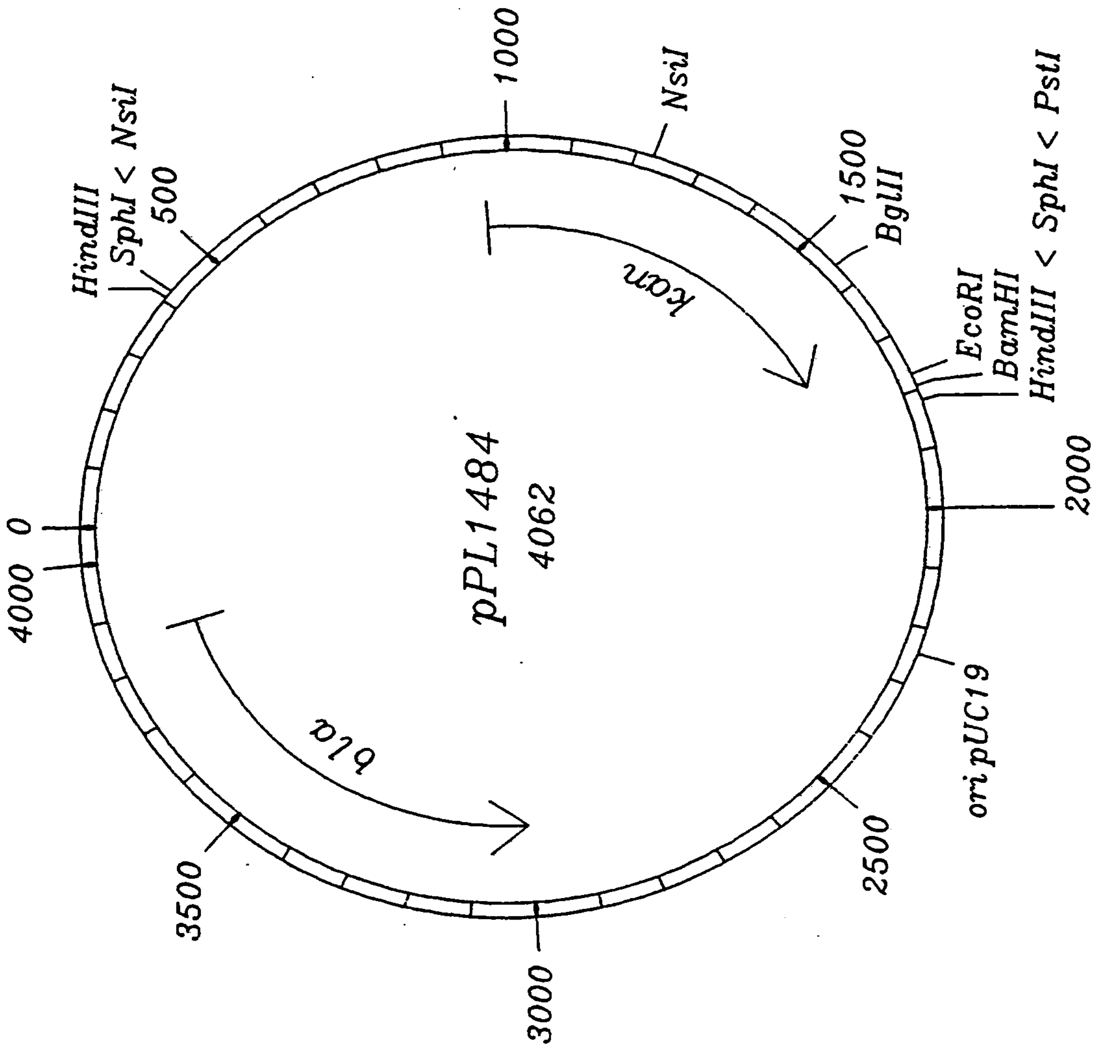


Fig. 12

REPLACEMENT SHEET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4062
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NsiI
1500
BglII
EcoRI
BamHI
HindIII < SphI < PstI
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ori pUC19
2500
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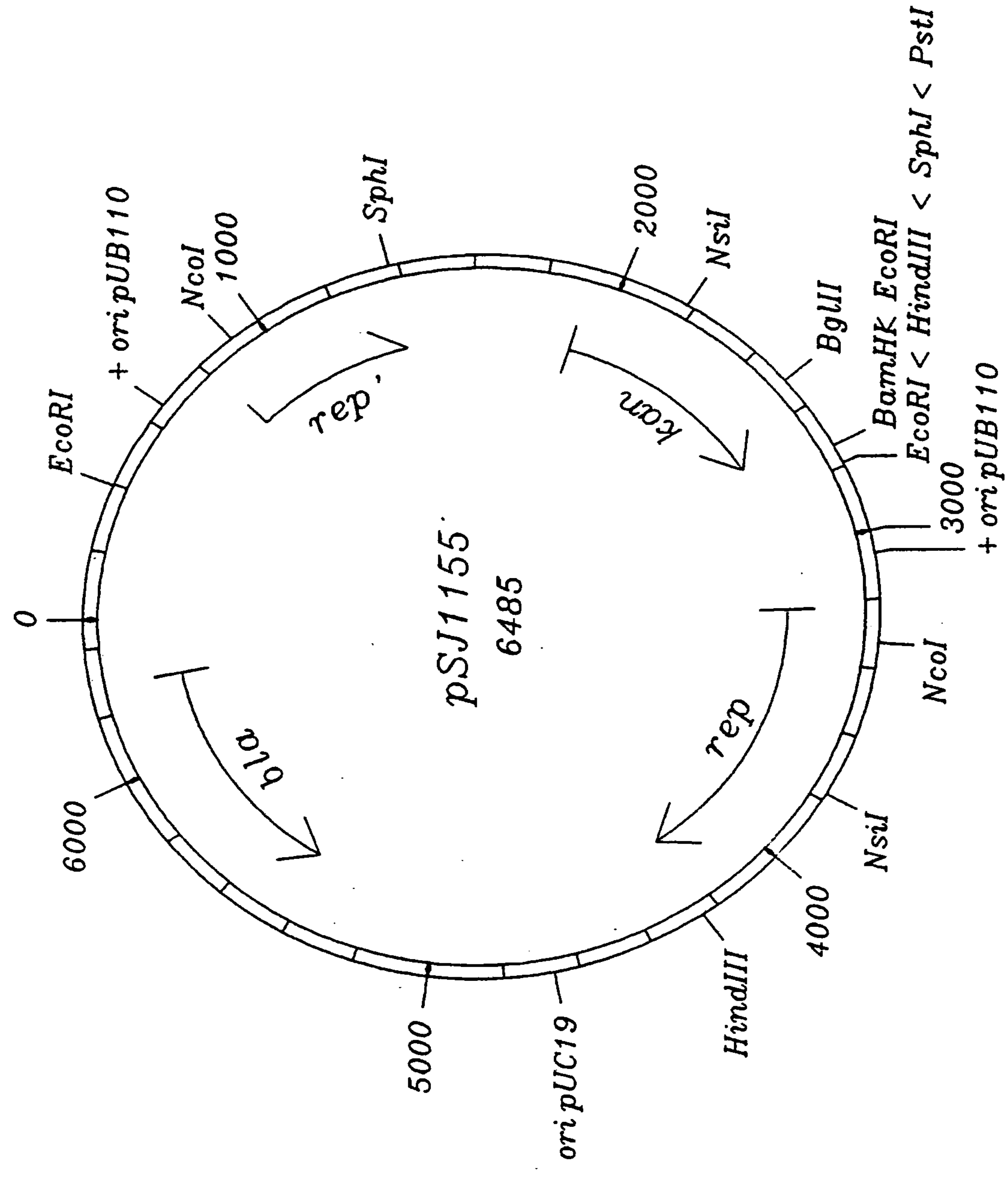
3500
4000
0
HindIII
SphI < NsiI
500
kan
bla

Fig. 13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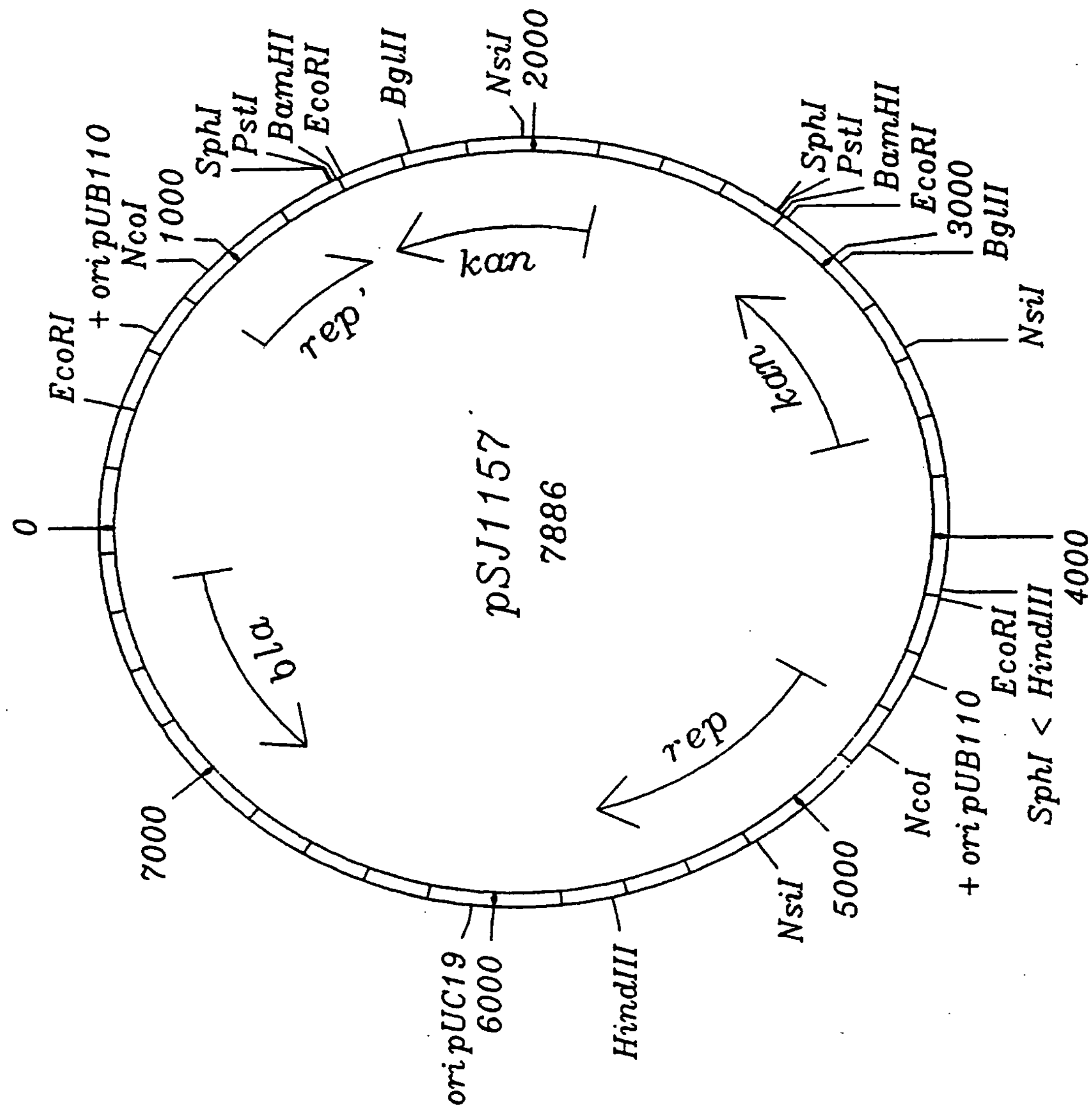
6485
rep'
kan
rep
bla
0
EcoRI
+ ori pUB110
NcoI
1000
SphI
2000
NsiI
BglII
BamHK EcoRI
EcoRI < HindIII < SphI < PstI
3000
+ ori pUB110
NcoI
NsiI
4000
HindIII
ori pUC19
5000
6000

Fig. 14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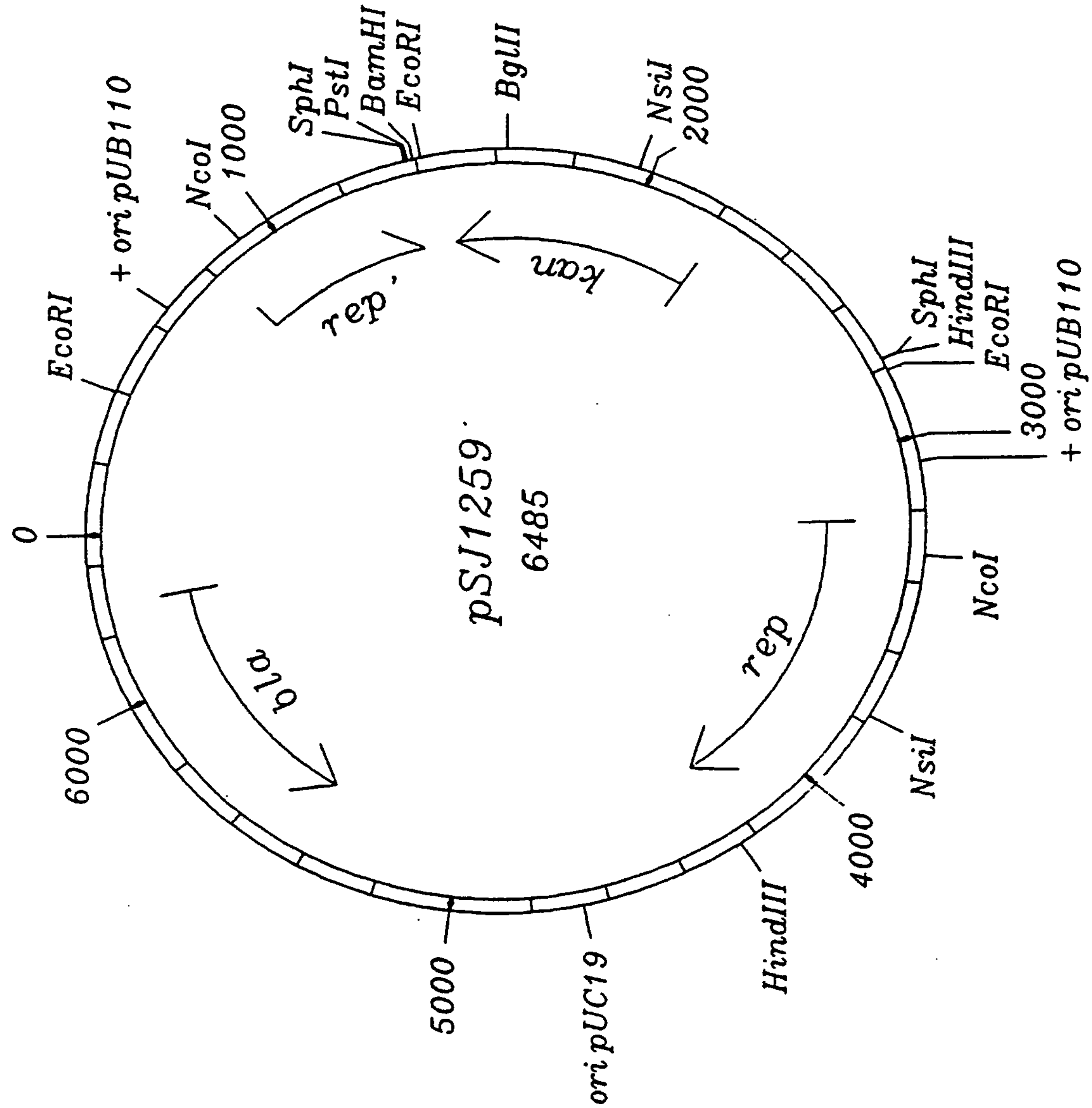
SphI
PstI
BamHI
EcoRI
BglII
NsiI
2000
SphI
PstI
BamHI
EcoRI
3000
BglII
NsiI
4000
EcoRI
HindIII
SphI < HindIII
+ ori pUB110
NcoI
5000
NsiI
HindIII
6000
ori pUC19
7000
kan
rep'
kan
rep
bla

Fig. 15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+ ori pUB110
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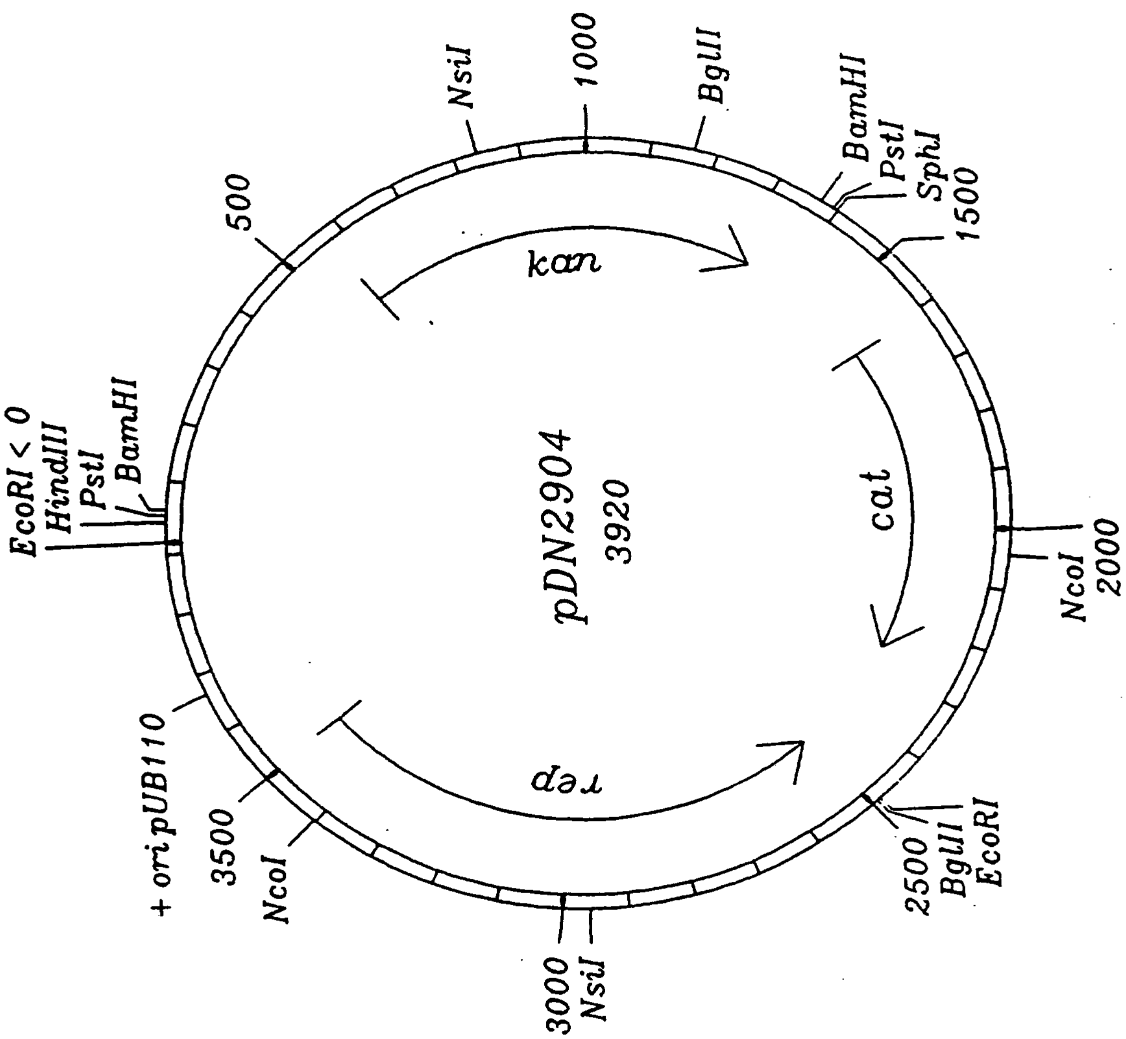
SphI
PstI
BamHI
EcoRI
BglII
NsiI
2000
SphI
HindIII
EcoRI
3000
+ ori pUB110
NcoI
NsiI
4000
HindIII
ori pUC19
5000
6000
rep'
kan
rep
bla

Fig. 16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cat
rep
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500
1500
2000
2500
3000
3500
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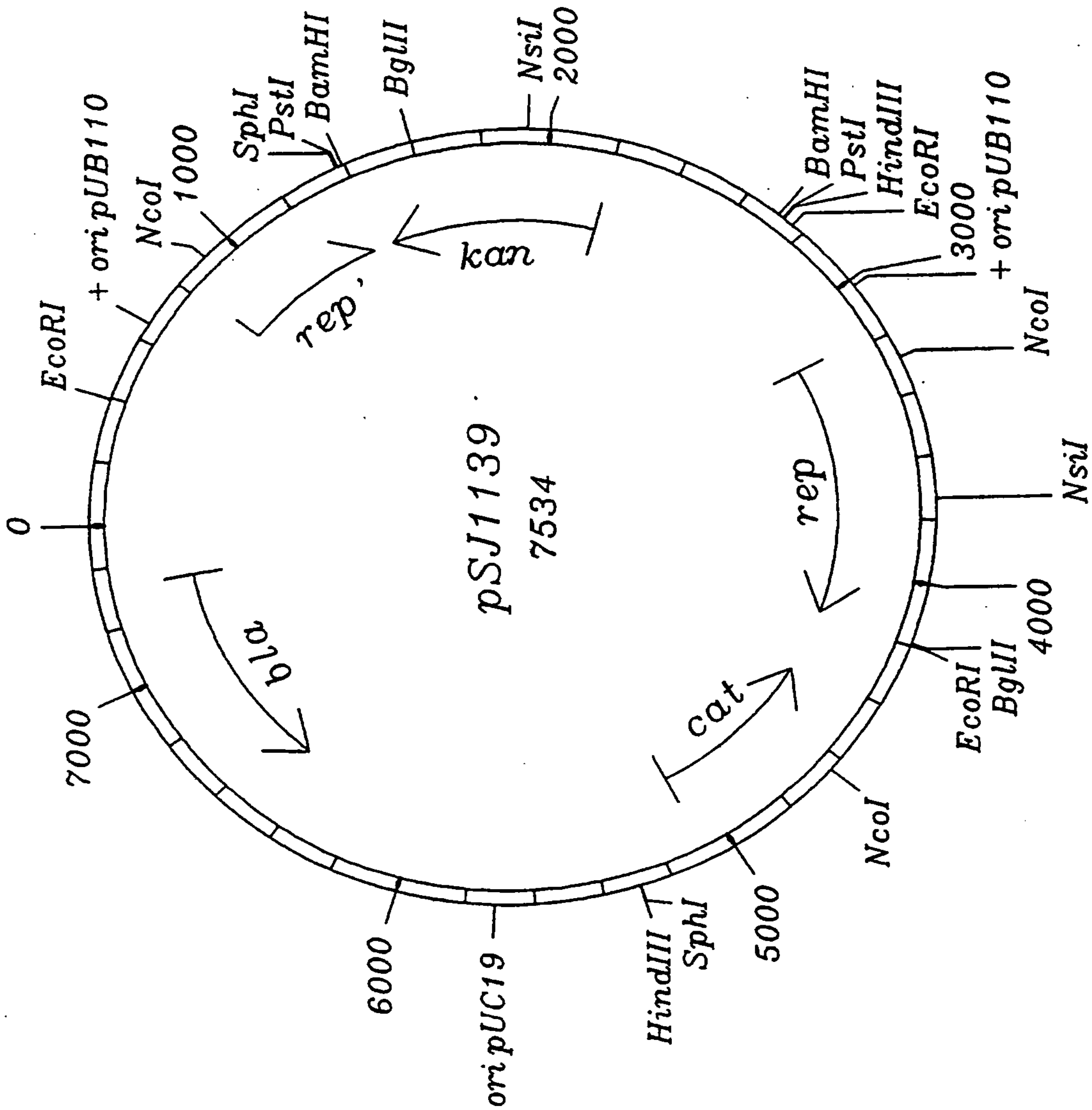
BglII
BamHI
PstI
SphI
NcoI
BglII
EcoRI
NsiI
NcoI
+ ori pUB110
EcoRI < 0
HindIII
PstI
BamHI

Fig. 17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cat
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PstI
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2000
BamHI
PstI
HindIII
EcoRI
3000
+ ori pUB110
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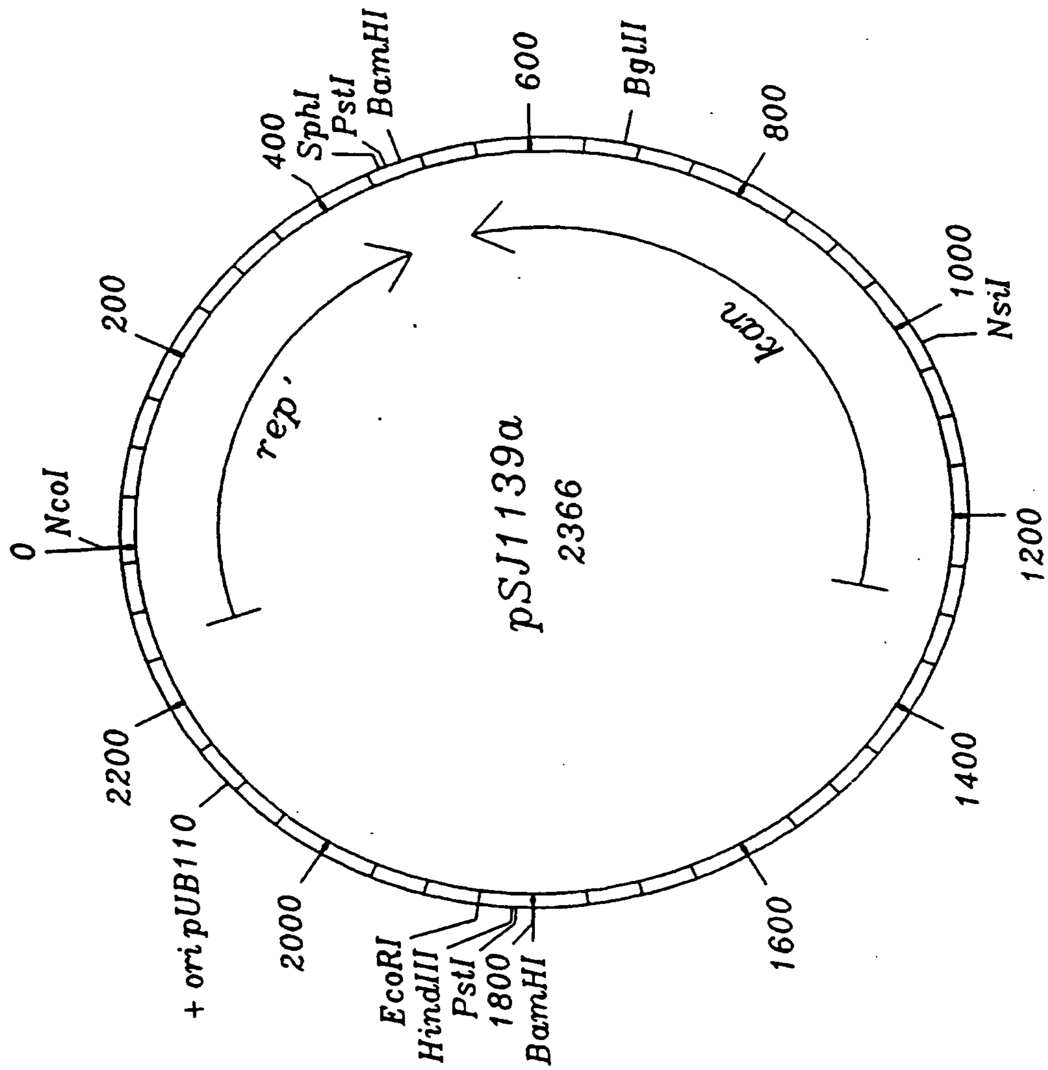
NsiI
4000
EcoRI
BglII
NcoI
5000
HindIII
SphI
ori pUC19
6000
7000

Fig. 18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2366
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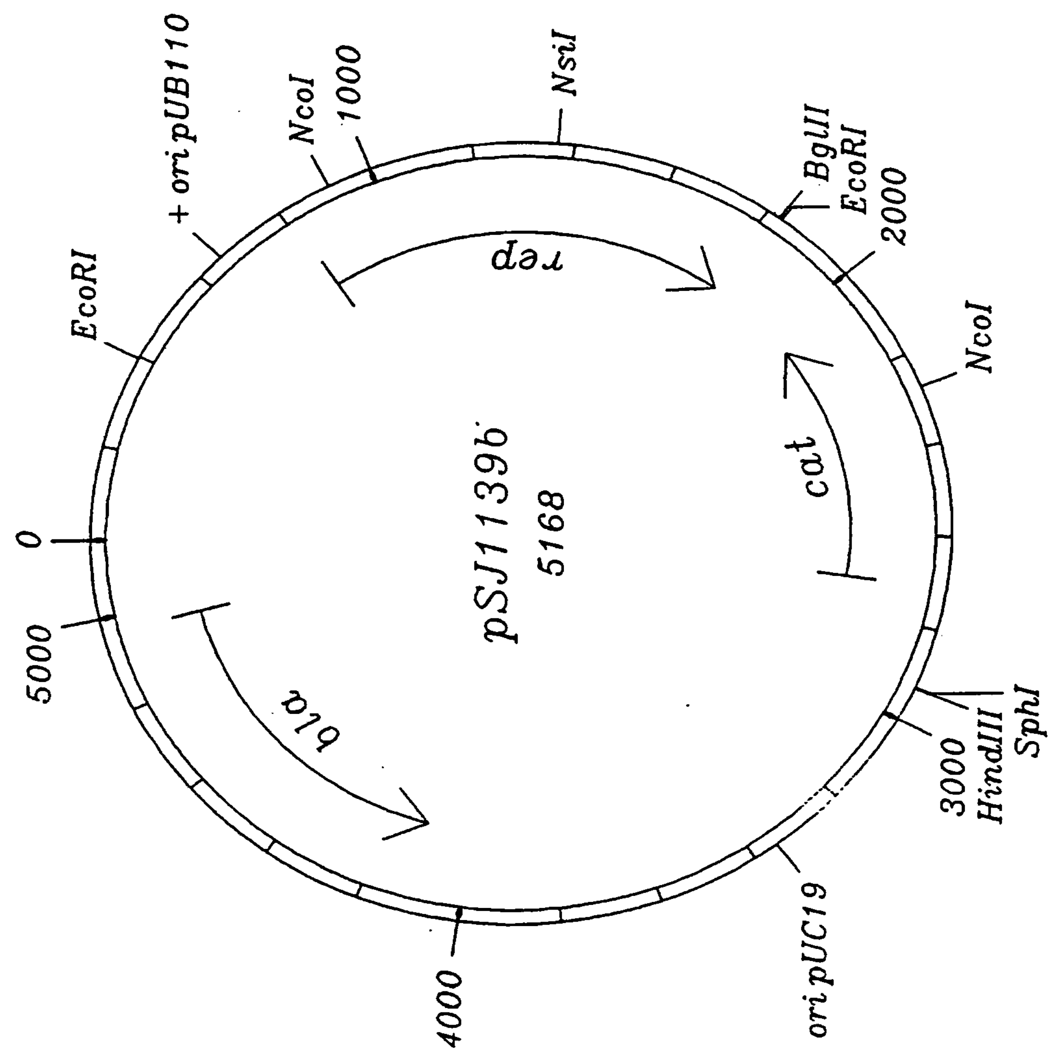
200
400
SphI
PstI
BamHI
600
BglII
800
1000
NsiI
1200
1400
1600
1800
BamHI
PstI
HindIII
EcoRI
2000
+ ori pUB110
2200

Fig. 19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BglII
EcoRI
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NcoI
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HindIII
SphI
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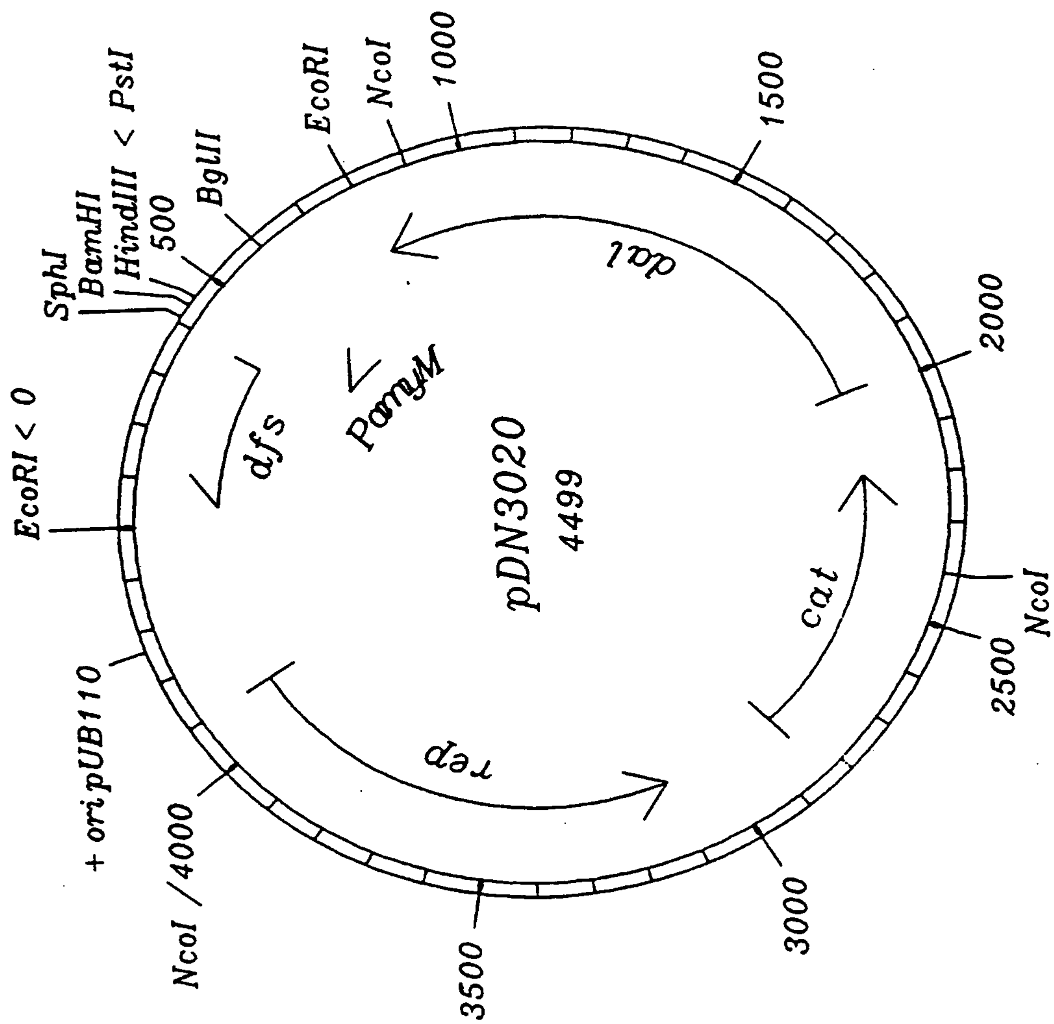
5000
0
EcoRI
+oripUB110
NcoI
1000

Fig. 20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dal
dfs
PamyM
cat
rep
SphI
BamHI
HindIII < PstI
500
BglII
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1000
1500
2000
NcoI
2500
3000
3500
NcoI /4000
+ ori pUB110
EcoRI < 0

Fig. 21

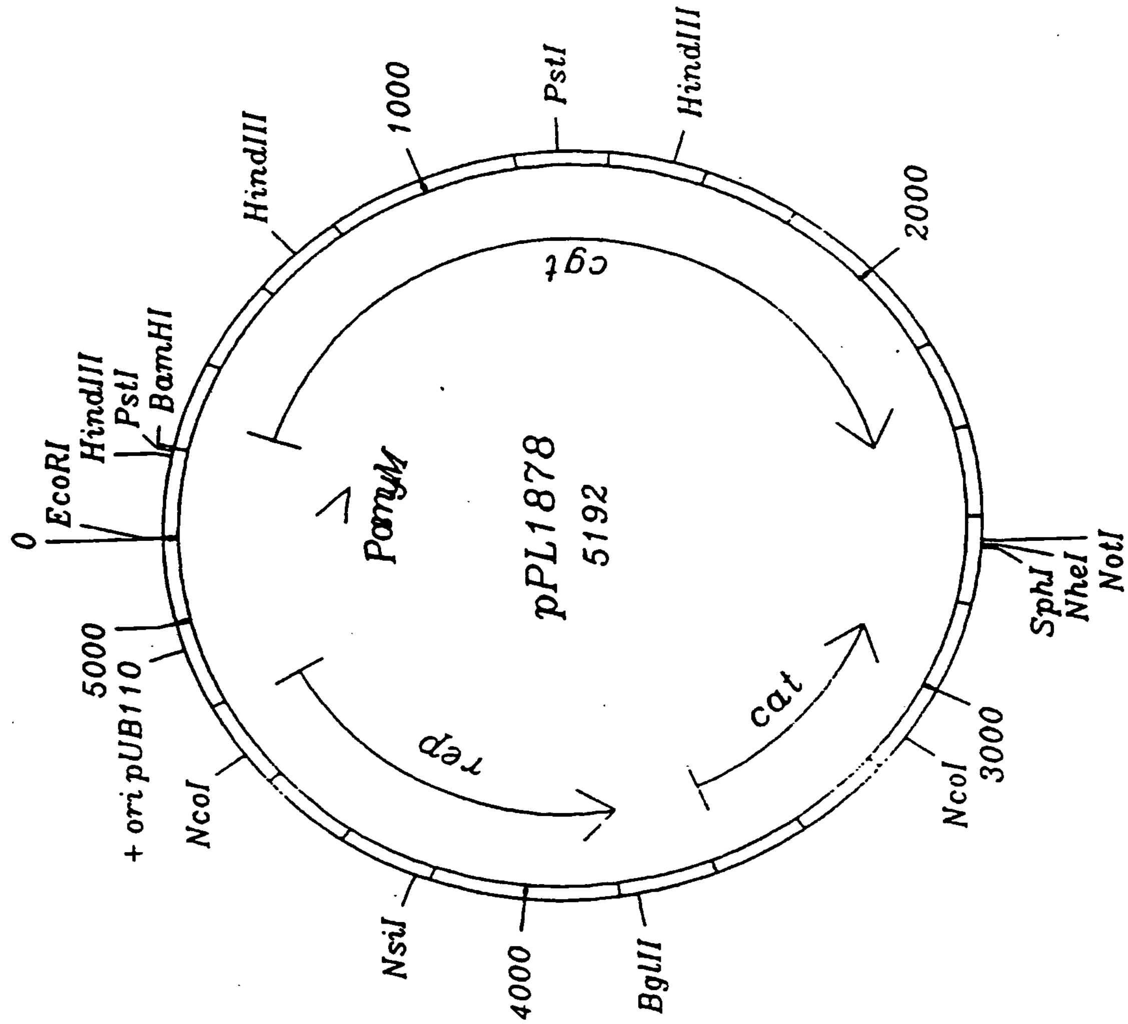

Fig. 22

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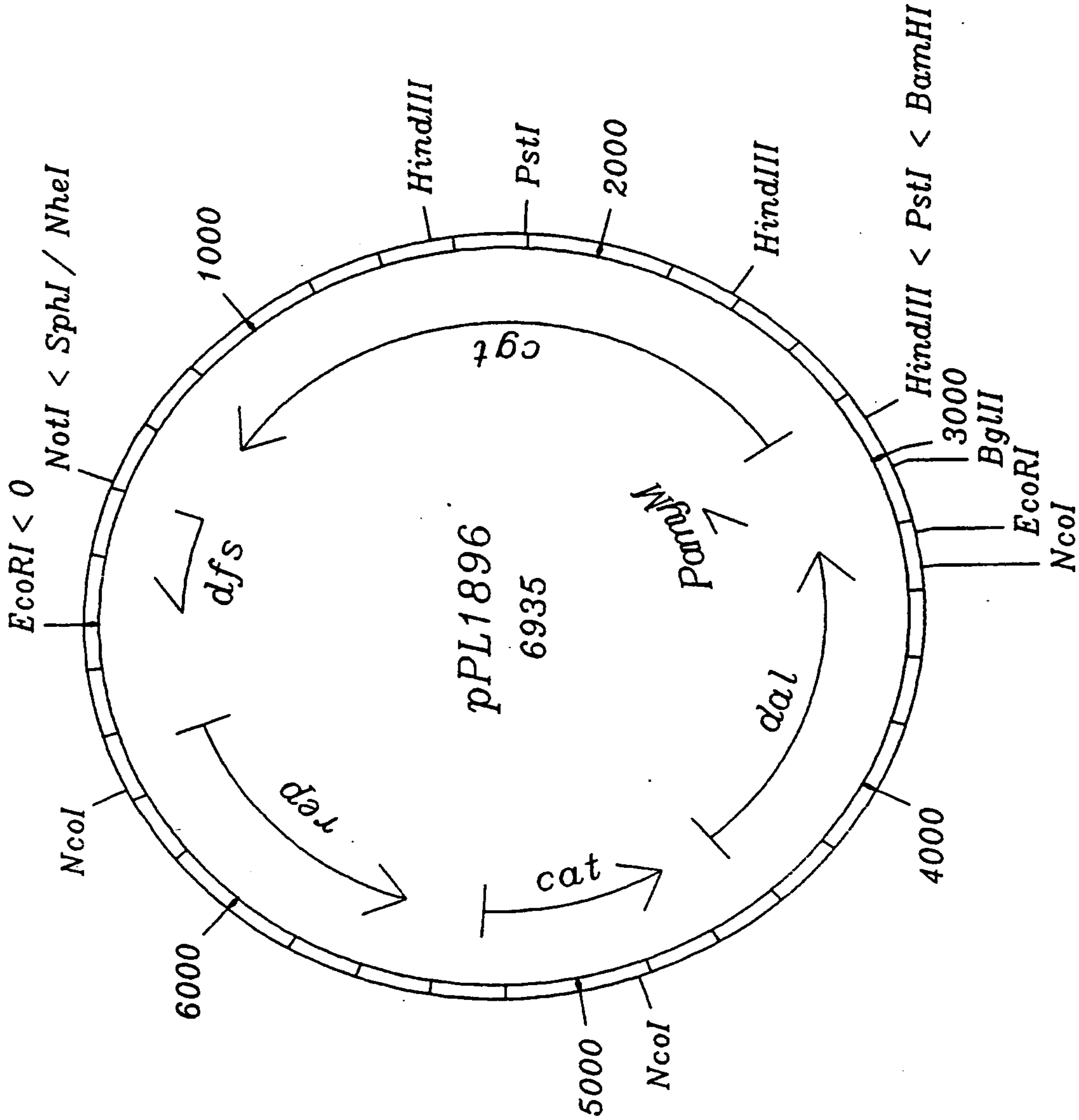
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6935
cgt
dfs
Pamy M
dal
cat
rep
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NotI < SphI / NheI
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HindIII
PstI
2000
HindIII
HindIII < PstI < BamHI
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BglII
EcoRI
NcoI
4000
NcoI
5000
6000
NcoI

Fig. 23

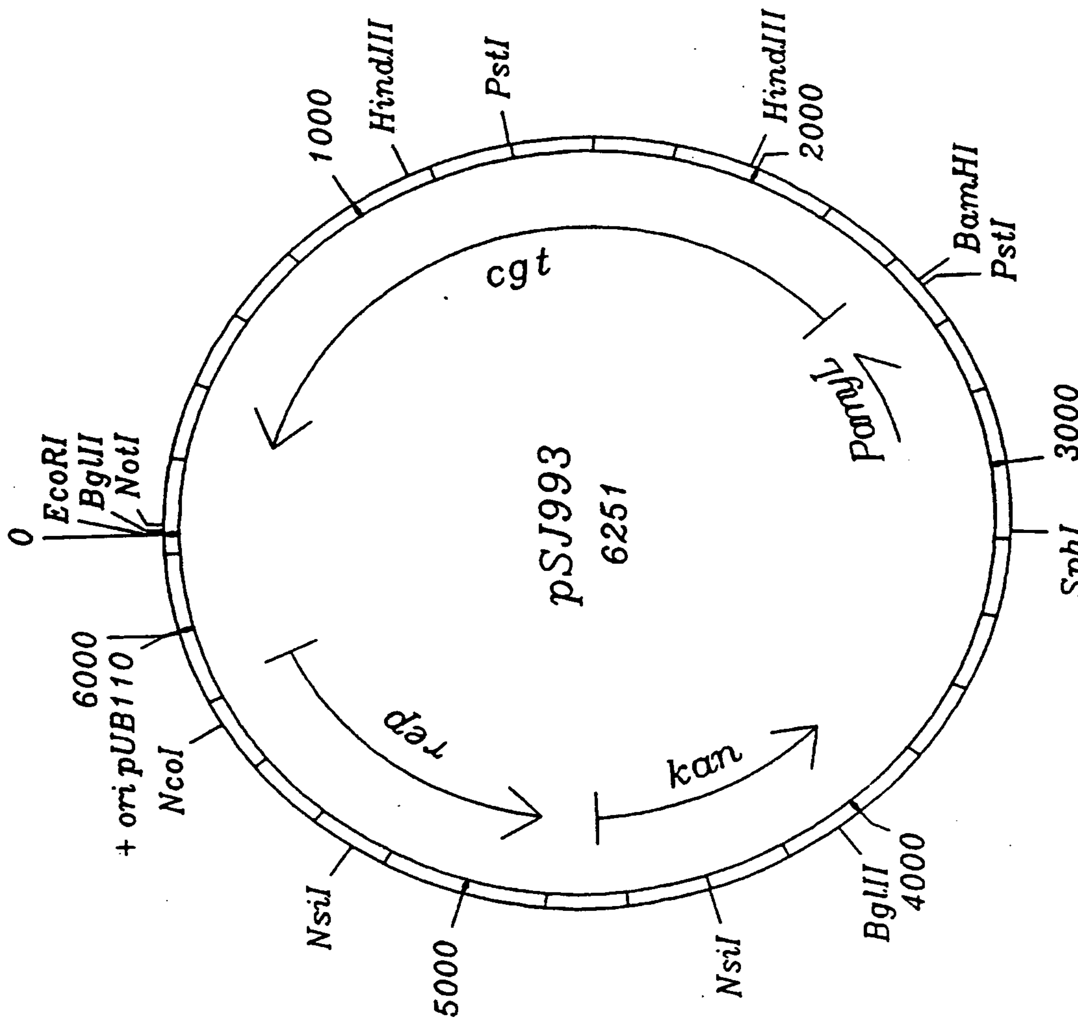
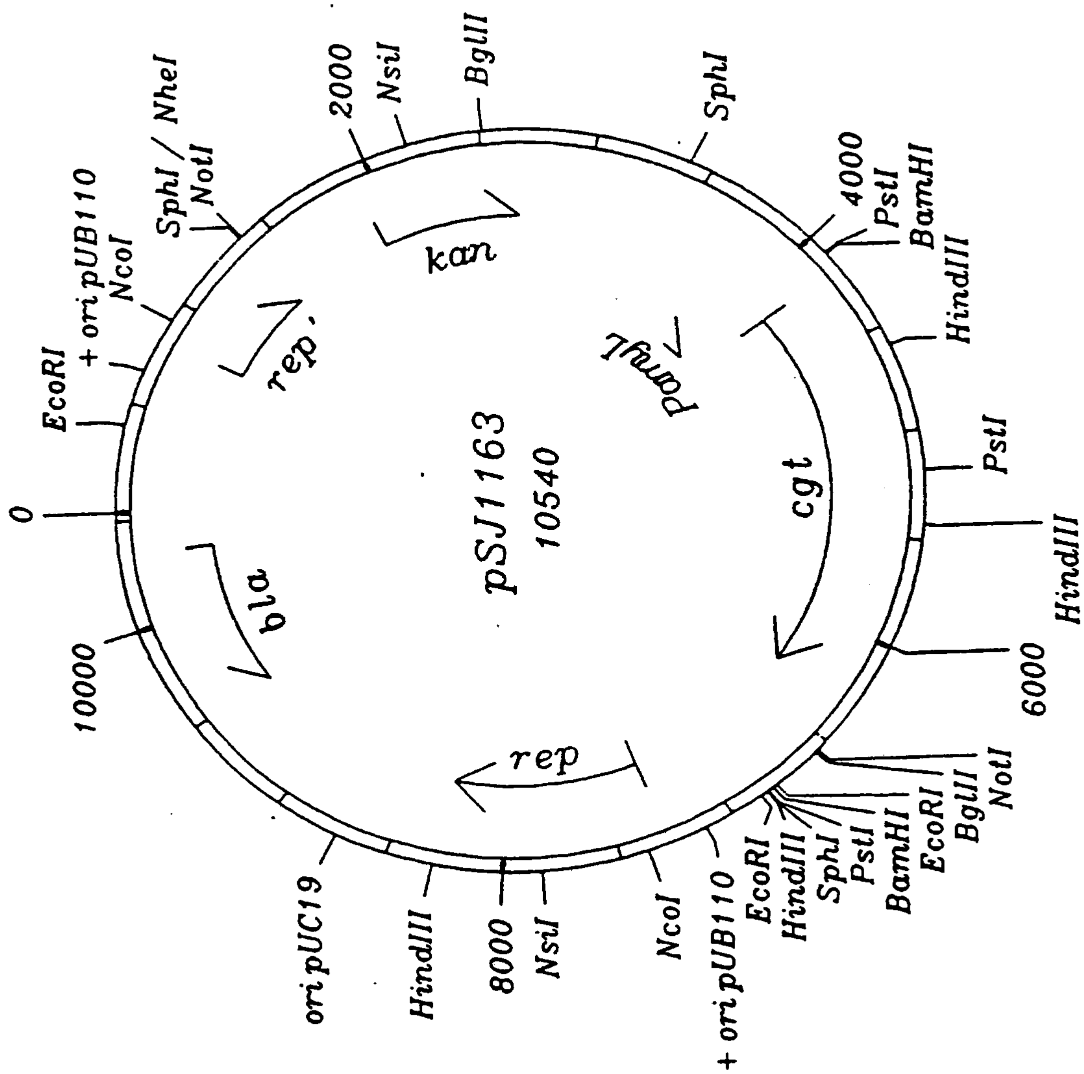


Fig. 24

REPLACEMENT SHEET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SphI / NheI
NotI
2000
NsiI
BglII
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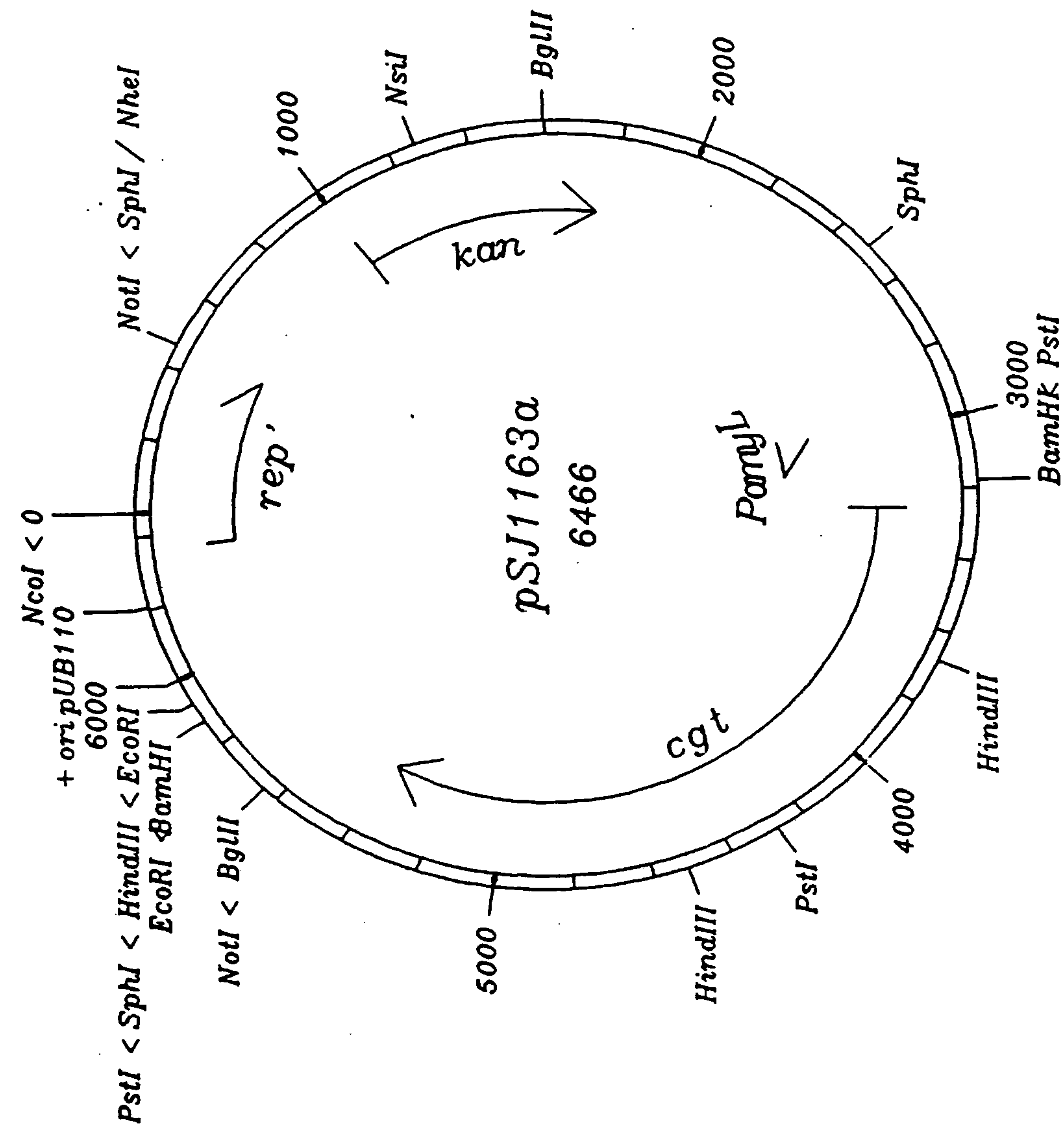
PstI
BamHI
HindIII
PstI
HindIII
6000
NotI
BglII
EcoRI
BamHI
PstI
SphI
HindIII
EcoRI
+ ori pUB110
NcoI
NsiI
8000
HindIII
ori pUC19
10000
kan
rep.
pamyL
cgt
rep
bla

Fig. 25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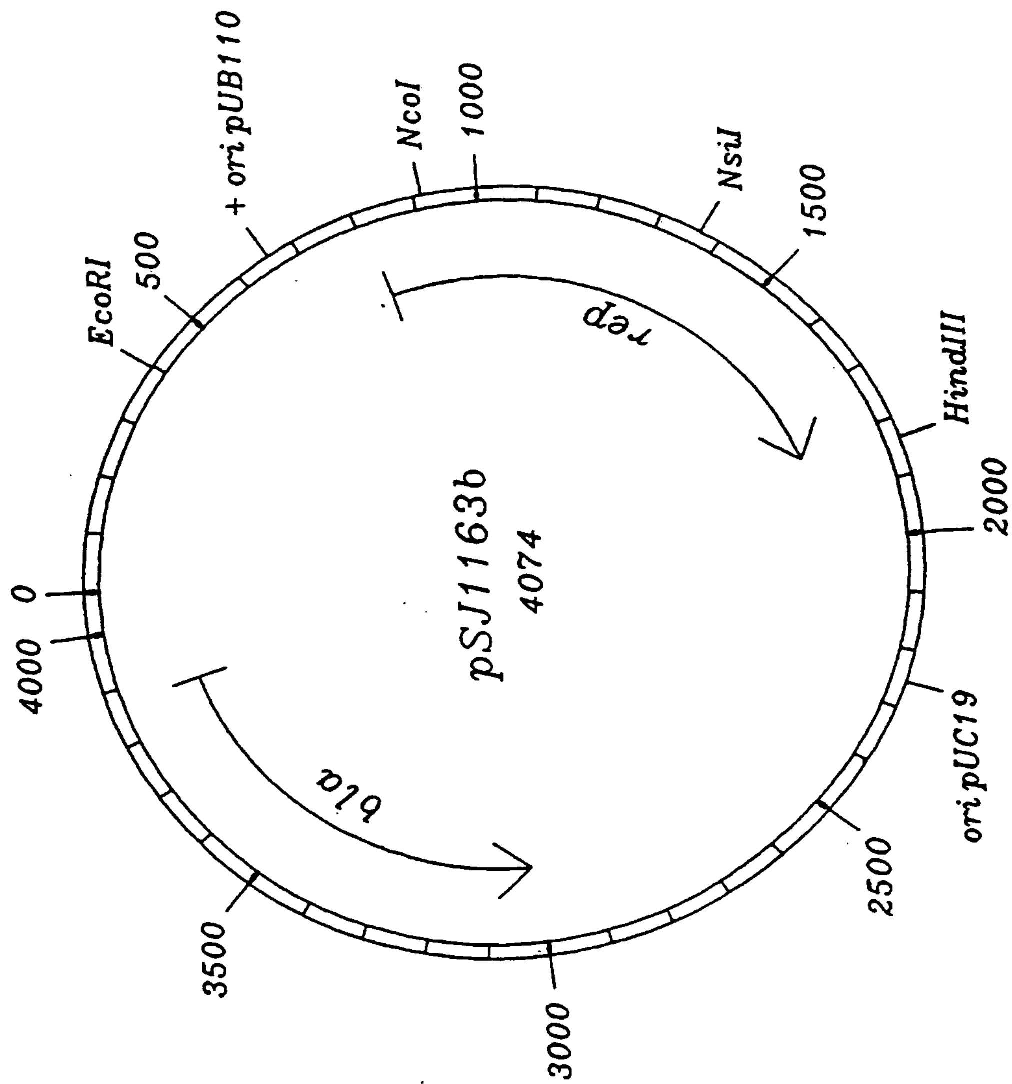
6466
kan
rep'
PamyL
cgt
BglII
2000
NsiI
1000
NotI < SphI / NheI
SphI
3000
BamHK PstI
NcoI < 0
+ ori pUB110
6000
PstI < SphI < HindIII < EcoRI
EcoRI BamHI
NotI < BglII
5000
HindIII
PstI
4000
HindIII

Fig. 26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rep
bla
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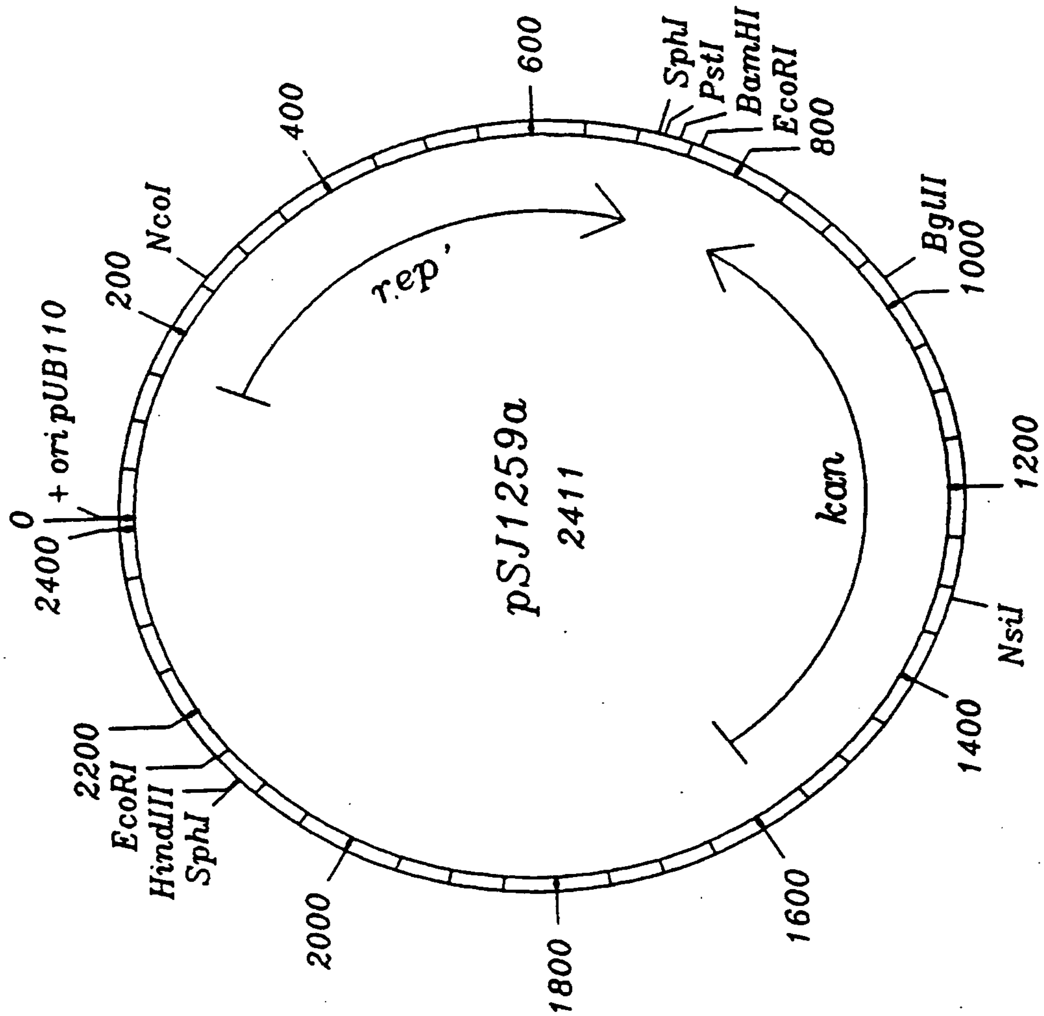
1000
NsiI
1500
HindIII
2000
ori pUC19
2500
3000
3500
4000
0

Fig. 27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rep'
kan
+ ori pUB110
NcoI
SphI
PstI
BamHI
EcoRI
BglII
NsiI
EcoRI
HindIII
SphI
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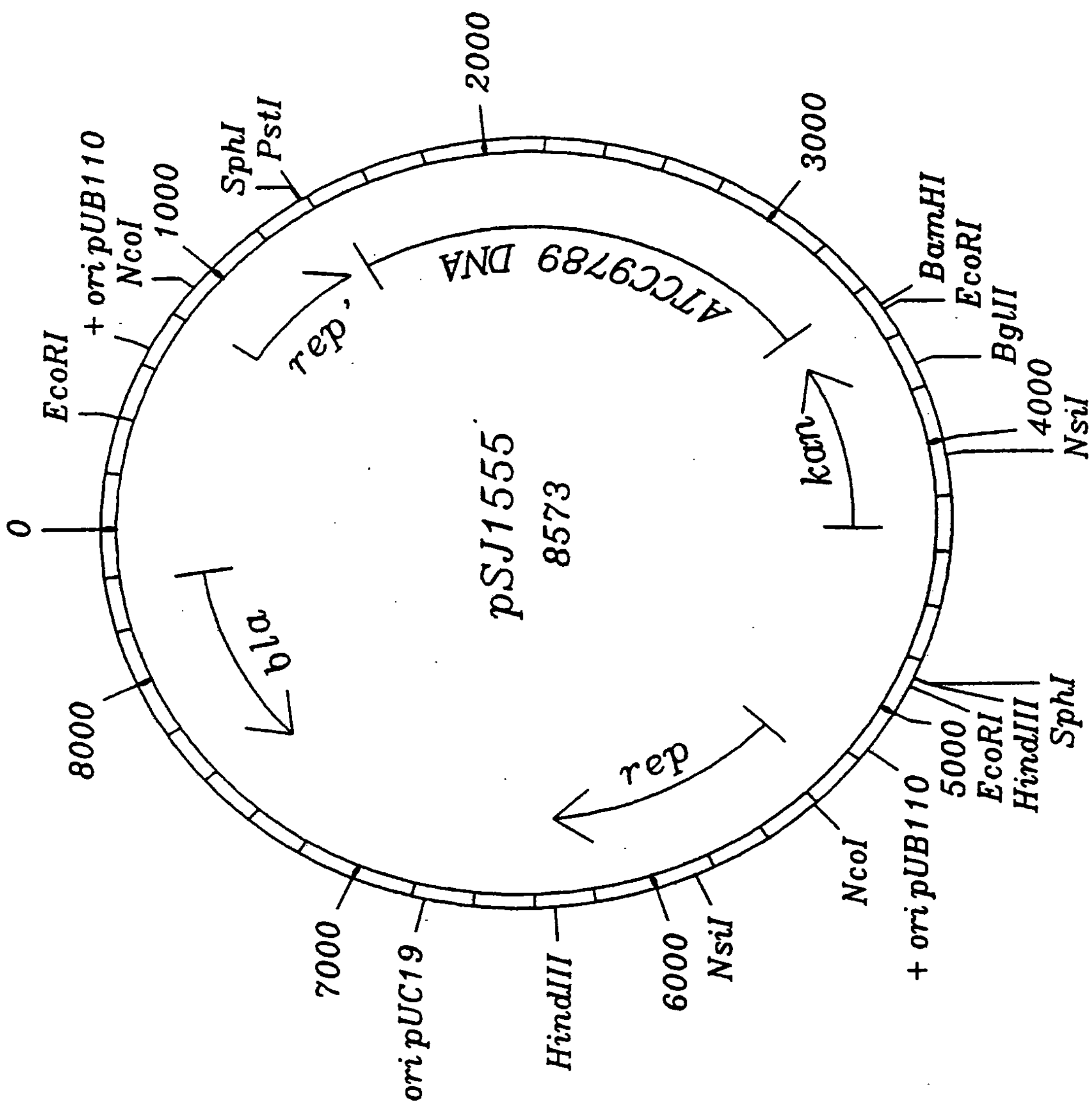
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400

Fig. 28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2000
3000
4000
5000
6000
7000
8000
EcoRI
+ ori pUB110
NcoI
SphI
PstI
BamHI
EcoRI
BglII
NsiI
SphI
HindIII
EcoRI
NcoI
+ ori pUB110
NsiI
HindIII
ori pUC19
rep'
ATCC9789 DNA
kan
rep
bla

Fig. 29

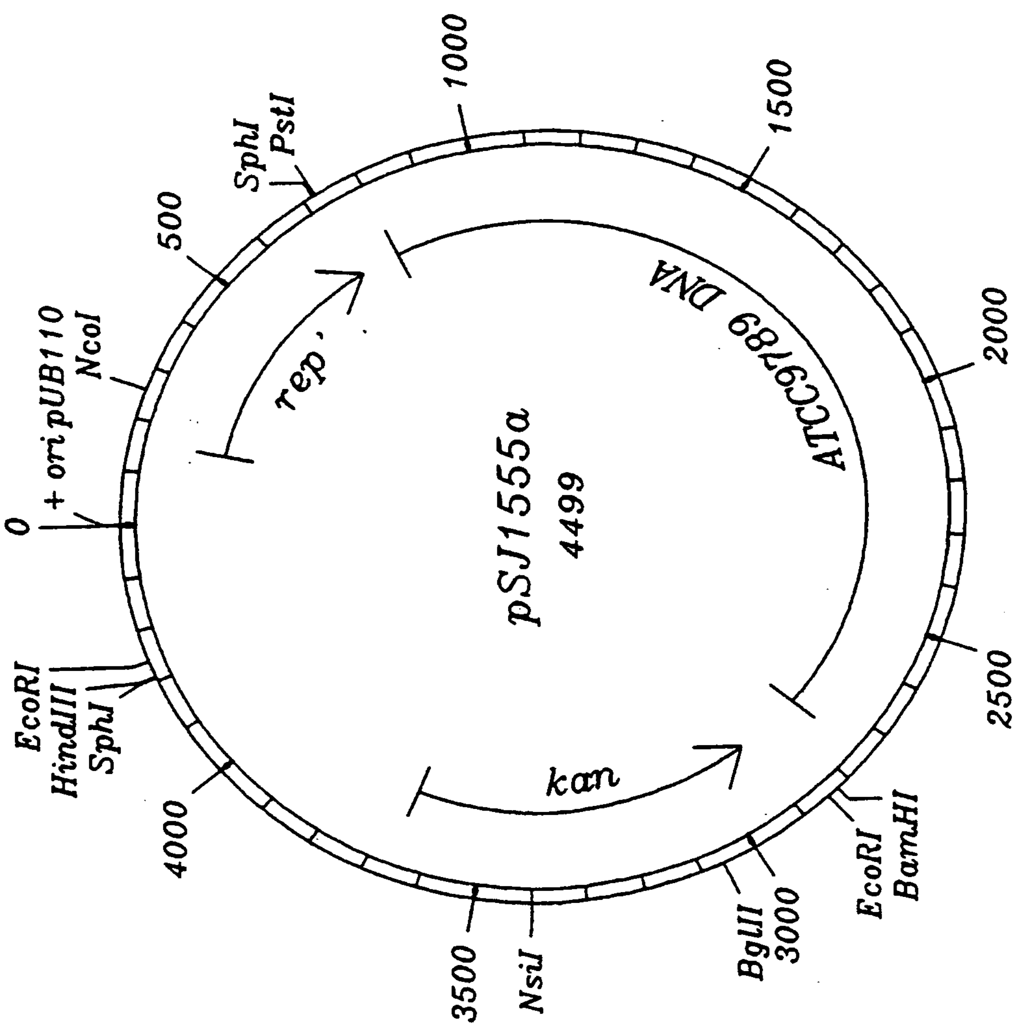
pSJ1555a
4499
0
+ ori pUB110
NcoI
500
SphI
PstI
1000
1500
2000
2500
ATCC9789 DNA
rep.
kan
EcoRI
BamHI
BglII
3000
NsiI
3500
4000
EcoRI
HindIII
SphI

Fig. 30

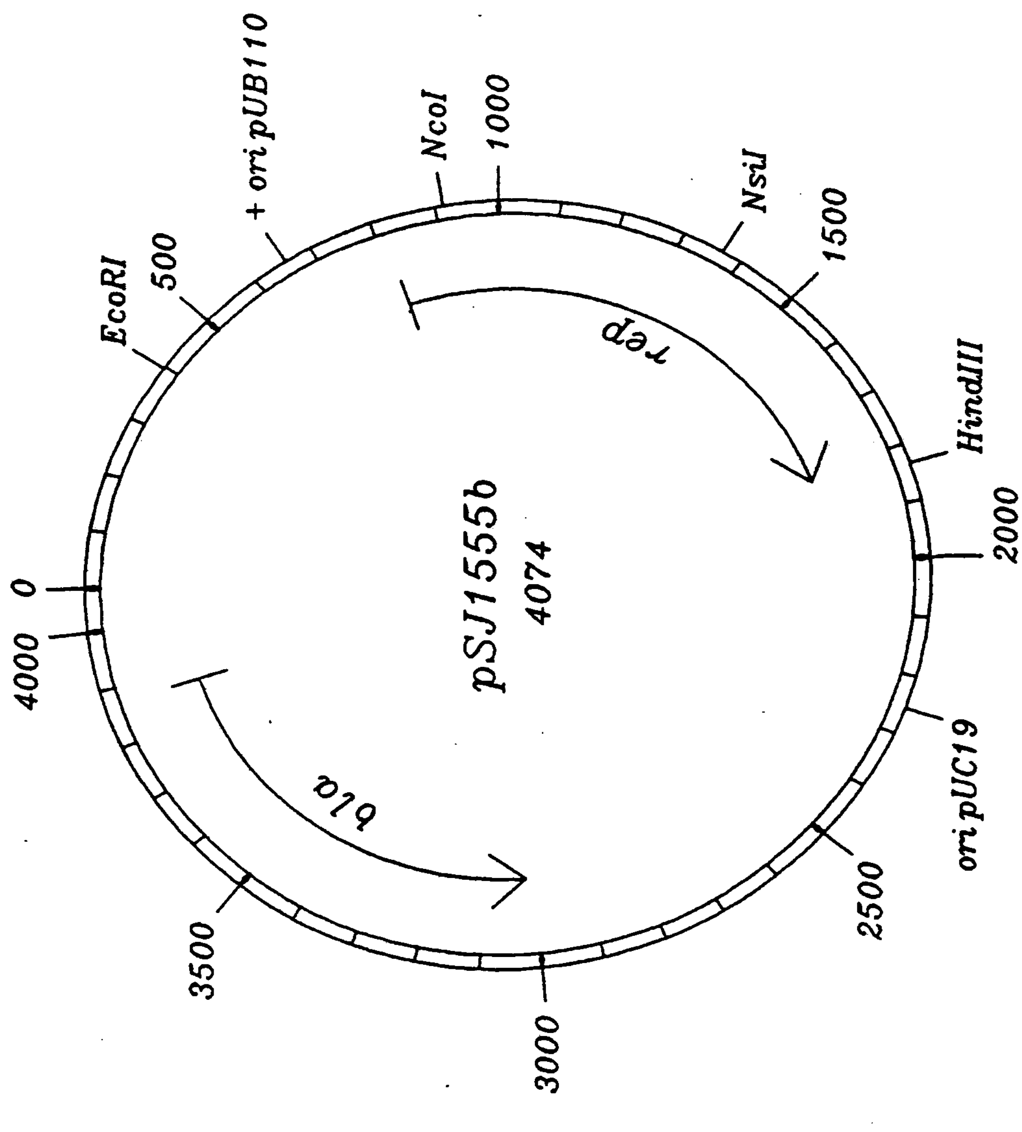
pSJ1555b
4074
rep
bla
EcoRI
+ ori pUB110
NcoI
NsiI
HindIII
ori pUC19
0
500
1000
1500
2000
2500
3000
3500
4000

Fig. 31

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00332

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 15/68, 15/63

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	Journal of Bacteriology, Vol. 171, No. 5, May 1989 Michael Young et al.: "Stability of Reiterated Sequences in the Bacillus subtilis Chromosome", see page 2653 - page 2656 --	1-17
X	US, A, 4631257 (DAVID H. GELFAND) 23 December 1986, see column 3 lines 37-62 --	1-4,43-44
A	US, A, 4401761 (JACK J. MANIS ET AL.) 30 August 1983, see the whole document --	1-17,43-44

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th March 1991	1991 -03- 19
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Yvonne Siösteen Yvonne Siösteen

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 90/00332

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-02-28
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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